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GROWTH AND ISOLATION OF *YERSINIA ENTEROCOLITICA*

by

MYRNA C. MUNGAL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

GROWTH AND ISOLATION OF *YERSINIA ENTEROCOLITICA*

submitted by MYRNA C. MUNGAL

in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The cardinal growth temperatures of three cultures of *Yersinia enterocolitica* were investigated in trypticase soy broth (TSB, pH 7.0) using a temperature gradient incubator and refrigerated water baths. Minimum growth temperature varied between 0.5 and 1.4°C while the maximum was recorded at 42°C. Optimum growth temperature for all cultures was found to be 32°C. Subsequent studies, using *Y. enterocolitica* Serotype 3, included a study on growth in TSB at pH 6 and 5, growth in TSB with added sodium chloride, and growth in a minimal medium, Glucose Salts medium, at pH 7, 6 and 5.

Growth responses of *Y. enterocolitica*, selected *Enterobacteriaceae* and a psychrophilic pseudomonad were determined at refrigerated temperatures using commercially available enteric media. *Y. enterocolitica* strains and the *Pseudomonas* sp. showed good growth on Violet Red Bile Glucose agar incubated at 6°C for 5 days; but *Y. enterocolitica* strains were found to be sensitive to Salmonella-Shigella (SS) and Brilliant Green (BG) agars at low temperature incubation. The use of selective enteric media at low temperature would appear to be a useful aid in the isolation of this organism from food products.

Viable counts of *Y. enterocolitica* inoculated in raw pork, as determined from representative, confirmed colonies picked from trypticase soy agar plates, increased over a 12-day period at 0, 5 and 10°C. Results obtained by low temperature isolation on Violet Red Bile Glucose agar were 50-80% lower than those obtained on TSA.

The potential public health hazard of *Y. enterocolitica*, as related to raw meat, is discussed.

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INTRODUCTION

Certain species of salmonellae, shigellae, staphylococci, clostridia and streptococci have long been recognized as important causes of food-borne bacterial disease. The clinical picture, epidemiology, pathogenicity, isolation, identification and control of the diseases are well documented. Within the last 20 years *Vibrio parahaemolyticus*, *Bacillus cereus* and aflatoxins have been added to the known food-borne hazards, largely as a result of improved laboratory methods and increased levels of surveillance. The most recent addition to the causative agents of food-borne bacterial disease is *Yersinia enterocolitica*.

Y. enterocolitica, a member of the *Enterobacteriaceae*, is a gram-negative, facultative anaerobic asporogenous bacillus 1.0 to 3.5 μm by 0.5 to 1.3 μm in size. Irrespective of biotype or serotype, this organism has been described as motile via peritrichous flagella at 25°C and non-motile at 37°C although a few strains have been reported motile or poorly motile when grown at 37°C (Niléhn, 1969). In broth cultures incubated at 25°C, *Y. enterocolitica* has been reported to be actively motile displaying a tumbling, spinning motion in contrast to the absence of motility when growth has occurred at 37°C. Occasionally, rare non-motile isolates may also be encountered (Bottone, 1977). The temperature range for growth is reported to be between -2 and 45°C with an optimum of 30-37°C (Bergey's Manual of Det. Bact., 1974). All strains are non-pigmented and not encapsulated.

The genus name *Yersinia* was proposed by Van Loghem in 1944 in honor of the French bacteriologist, A.J.E. Yersin, who first isolated

the plague bacillus *Yersinia* (*Pasteurella*) *pestis* in 1894. In 1954, Thal proposed the inclusion of those organisms comprising the genus *Yersinia* (*Y.* [*Pasteurella*] *pestis* and *Y.* [*Pasteurella*] *pseudotuberculosis*) into the family *Enterobacteriaceae*. The validity of this proposal was supported by Sneath and Cowan (1958) and Talbot and Sneath (1960) who showed a high degree of similarlity between these species and members of the family *Enterobacteriaceae*.

Working with the so-called "*Pasteurella* X", Smith and Thal (1965) substantiated Frederiksen's (1964) assignment of this species to *Yersinia enterocolitica* and further highlighted its similarity to *Y. pseudotuberculosis* and its distinctiveness to *Y. pestis* (Bottone, 1977). *Y. enterocolitica* is currently classified in the genus *Yersinia* within the family *Enterobacteriaceae* - a change that is reflected in the 8th edition of Bergey's Manual for Determinative Bacteriology (1977).

Reasons for placing *Yersinia* in the family *Enterobacteriaceae* could be summarized:

1. Both groups are fermenters, i.e. utilize carbohydrates by fermentative pathways;
2. Both reduce nitrate to nitrite;
3. Two of the three *Yersinia*, when motile (at 25°C, but not at 37°C) have peritrichous flagella like the *Enterobacteriaceae*;
4. Both groups are insensitive to bile salts - i.e. they grow on MacConkey agar (Darland *et al.*, 1974);
5. Antigenic relationship between the two groups exist (Diaz, 1973);
6. The DNA base composition of *Yersinia* is close to that of several *Enterobacteriaceae* (Domaradskij *et al.*, 1973);

7. DNA/DNA homology studies support the relative closeness of the 2 groups (Ritter *et al.*, 1966).

The first recognized description of *Y. enterocolitica* in human pathology was rendered in the U.S. in 1939 by Schleifstein and Coleman who isolated an unidentified microorganism pathogenic for man similar to *Bacterium liguieri* and *Pasteurella pseudotuberculosis* and called it *Bacterium enterocoliticum*. It was first isolated from two patients with cervical adenitis and three with acute enteritis and terminal ileitis. Six additional cases in the U.S. were reported from 1940-47; no other cases of human infection were recognized in the U.S. for the next 21 years when a case of *Y. enterocolitica* meningitis with panophthalmitis was reported in Missouri by Sonnenwirth (1968).

European investigators in the meantime were devoting their efforts to investigations of the occurrence of this unnamed species in various animal hosts, which led to the recognition of the pig, hare and chinchilla as important reservoirs. In these reports, the organism was designated *Pasteurella pseudotuberculosis* type b, *Pasteurella* X and *Germe* X. In 1949, Hässig *et al.* isolated strains of bacteria which they identified as human *Pasteurella pseudotuberculosis* but later Knapp and Thal (1973) concluded that these Hässig strains did not belong to that species but to one closely related. In the early 1960's, clinical bacteriologists obtained a number of new isolates of bacteria which they described as "*Pasteurella pseudotuberculosis*", "*Pasteurella pseudo-tuberculosis*-like organisms and "*Pasteurella* Y". By 1965, Wauters and Mollaret and Winblad *et al.* in 1966 reported on the isolation of *Y. enterocolitica* from human sources. In 1964, Frederiksen found

Pasteurella X and the Hässig strains to be similar to the strain isolated by Schleifstein and Coleman, and proposed a new name for the species - *Yersinia enterocolitica*. The confusion surrounding the naming and classification of this organism no doubt contributed to the slow progress in recognizing and establishing its role in human disease.

During the next few years, reports of isolates from human sources continued to appear in some parts of the world, particularly in Europe. By 1970, 642 cases of yersiniosis were diagnosed, while in 1972, Mollaret had over 1000 cases recorded. Also in this year, the first isolations of *Y. enterocolitica* from human cases were described in Japan by Zen-Yoji and Maruyama and in South Africa by Rabson and Koornhoj. At present, over 5,800 isolates of *Y. enterocolitica* have been reported and indications are that the numbers will continue to increase.

Culturally, *Y. enterocolitica* grows well on a variety of artificial media utilized in the laboratory; however, some strains show a marked degree of variability in their ability to develop on these substrates. For example, while most strains belonging to Serotypes 3, 8 and 9 (the most frequently encountered serotypes in human infections) grow on Endo, Eosin Methylene Blue (EMB), MacConkey, Desoxycholate, Hektoen-Enteric (HE), Salmonella-Shigella (SS), and Xylose-lysine-deoxycholate (XLD) agars, rhamnose-positive strains of Serotypes 14, 16 and 17 fail to develop on HE and SS agars and grow poorly on XLD, even when incubated at 22°C (Bottone *et al.*, 1974; Chester and Stotzky, 1976).

On enteric media capable of supporting the growth of *Y. enterocolitica* strains (i.e. Endo, S.S. and MacConkey) colonies usually develop slowly. After incubation for 24 hrs at 37° or 22°C, colonies

are pinpoint to 0.05 mm in diameter as contrasted to colonies of *E. coli* or *Salmonella* sp. which achieve a colony size of 4 to 5 mm during the same interval. After incubation for 48 hrs at these two temperatures, *Y. enterocolitica* colonies approach a diameter of 0.5 to 2.5 mm. Because of their slower growth rate at 37° and 22°C, these colonies may easily be overlooked when present in small numbers and surrounded by colonies of other enteric organisms as in the case when attempting the recovery of *Yersinia* sp. from food specimens. Hence, to facilitate the isolation of this organism, Wauters (1970) and Toma (1973) have suggested scanning the enteric agar surfaces with the aid of a stereoscopic microscope using oblique illumination. Pinpoint colonies may then be selected for isolation as potential *Y. enterocolitica* colonies.

Two modified media for the recovery of *Y. enterocolitica* from foods have been described by Lee (1975). Because Tween 80 is heat stable, soluble, and easy to use, it is incorporated into two commonly used media to facilitate the selection of *Y. enterocolitica* colonies. In one medium, 10g of Tween 80 and 0.2g of $CaCl_2$ are incorporated into one litre of MacConkey agar. After 48 hr of incubation at 25°C on this medium, the Tween-positive strains of *Y. enterocolitica* produce flat, wrinkled colonies surrounded by a zone of precipitate which imparts a sheen-like effect to the agar medium. Lee further suggests that while this medium is useful for detecting Tween-positive *Y. enterocolitica* of Biotypes 1 and 2, not all biotypes display this capability equally, and also other enteric organisms such as *Serratia liquifaciens* and some *Pseudomonas*

species may impart identical reactions. However, colonial characteristics of these enterics differ from those of *Y. enterocolitica*. The second medium described by Lee to be used in conjunction with cold enrichment procedures consists of incorporating 10g Tween 80, 10g sorbitol, 0.3g sodium dodecyl sulfate and 0.2g of $CaCl_2$ per litre of deoxyribonuclease (DNase) agar. Following sterilization and cooling to 50°C, 40 mg/L acridine orange and 25 mg/L of filter-sterilized triphenyl tetrazolium chloride are added to the DNase medium. On this medium, Lee reports that *Y. enterocolitica* colonies are typically translucent, colorless or pink with little or no Tween reaction or nuclease reaction when examined under long-wave ultraviolet light.

Y. enterocolitica survives very well in nature, especially at low temperatures (Morris and Feeley, 1976). Reports indicate that this organism will grow at 4°C and because of this, it has been suggested that cold temperature.(4° to 7°C) enrichment procedures would enhance the recovery of *Y. enterocolitica* from food sources (Paterson and Cook, 1963; Zen-Yoji *et al.*, 1972; Tsubokura *et al.*, 1973; Feeley *et al.*, 1976).

Presently, there are no standard laboratory methods for the isolation and enumeration of *Y. enterocolitica*. Various techniques have been used by investigators and found to yield good recovery of this organism. Feeley *et al.* (1976) in the "Compendium of Methods for the Microbiological Examination of Foods" prepared by the American Public Health Association (APHA) and edited by M.L. Speck, and Highsmith *et al.* (1977) recommend the following procedure for the isolation and identification of *Y. enterocolitica* from foods:

25g food sample added to a 225 ml 1/15 M sodium phosphate buffer, blended for 2 mins

Incubate 21 days 4°C

Plate 0.1 ml aliquots onto MacConkey and SS agar to obtain isolated colonies

Inoculate 0.1 ml into 10 mls magnesium chloride-malachite green - Carbinicillin medium 9 or Selenite F broth

Incubate 25°C, 48 hrs

Select 5 lactose negative colonies

SCREENING TESTS

TSI	25°C	overnight
LIA	25°C	overnight
UREA	25°C	24 hrs
Motility	25°C	24 hrs
	36°C	24 hrs

PRESUMPTIVE IDENTIFICATION TESTS

Oxidase	
Lysine decarboxylase	36°C
Arginine dihydrolase	36°C
Ornithine decarboxylase	25°C; 36°C
Phenylalanine deaminase	36°C
Indole	36°C

CONFIRMED TESTS 36°C

Lactose
Maltose
Sucrose
Melibiose
Raffinose
Rhamnose

OPTIONAL TESTS

biotype
serotype

Two of the more widely utilized cold enrichment procedures are those of Wetzler (1970) who recommended inoculating isotonic saline with or without 25 µg/ml potassium tellurite followed by subculture after 24 hrs at 4° to 7°C, and Paterson and Cook (1963) who utilized 0.067 M phosphate buffer (pH 7.6) at 4°C with subculture at intervals ranging up to 28 days. Greenwood *et al.* (1975) and Toma and Deidrick (1975) favoured Paterson and Cook's proposal since it yielded a greater percentage of *Yersinia* isolates. With cold temperature enrichment procedures, *Yersinia* species continue to multiply while other vegetative microbial species do not grow or are slowly killed, thereby enhancing recovery (Feeley *et al.*, 1976).

By 1960, *Y. enterocolitica* strains isolated from humans, animal and food sources, had been recognized to be biochemically heterogeneous. Typical isolates of this organism ferment a variety of carbohydrates anaerogenically. Glucose, sucrose, arabinose, galactose, mannitol, sorbitol, trehalose, xylose and cellobiose are all utilized at both 37° and 25°C within 24 hrs with little or no variation. Rhamnose, raffinose, dulcitol and adonitol are usually not utilized, while acid from salicin and esculin hydrolysis is strain variable. Maltose is utilized best at 22°C and lactose is rarely attacked within 48 hrs and usually requires an incubation period of up to 30 days in order to detect a visible acid pH change in the indicator (Nilé́hn, 1969).

Rapidity of utilization of any of the above substrates may vary slightly with temperature, but tests for acetylmethyl carbinol (Voges-Proskauer), growth in potassium cyanide, ornithine decarboxylase activity and motility are solely temperature dependent and are expressed

at 22° but absent at 37°C.

Y. enterocolitica lacks enzymatic activity for oxidase, gelatinase, phenylalanine deaminase, citratase, lysine decarboxylase and arginine dihydrolase, but possesses catalase, β-galactosidase, nitrate reductase type B and urease activities, while extracellular deoxyribonuclease is strain variable. It has been reported that urease production is readily demonstrated on Christensen's urea agar but is not obvious in the more highly buffered formulation of Rustigan and Stuart (1941). Urease activity may be delayed or even absent upon initial isolation, but usually following subculture and re-testing, urease production is evident within 5 hours (Niléhn, 1969; Bottone *et al.*, 1974).

Indole production in *Y. enterocolitica* strains thus isolated is variable (Coleman, 1950, Niléhn, 1969, Ahvonen, 1972; Knapp and Thal, 1973), with most European isolates being reported as indole negative as compared to the U.S. where most isolates are indole positive. Bottone *et al.* (1974) indicated that testing for indole production is dependent upon substrates utilized, incubation temperature and choice of reagent. Niléhn (1969) reported strong reactions with Kovac's reagent using tryptone broth incubated at 37°C and Ahvonen (1972) showed that all isolates of Serotype 9 were weakly indole positive, especially at 25°C.

Because of the biochemical heterogeneity that exists among strains of *Y. enterocolitica*, Niléhn, in 1969, first proposed arranging the strains according to their biochemical reactions into groups or "biotypes" (Table 1). Keeping methodologies and incubation temperatures constant, she investigated 330 *Y. enterocolitica* isolates and, on the basis of positive characters, devised 5 biotypes. In the following year,

TABLE 1

Biotypes of *Yersinia enterocolitica*

Tests	*Y. enterocolitica* biotypes													
	Niléhn					Wauters					Knapp and Thal			
	1	2	3	4	5	1	2	3	4	5	1	2	3[a]	4[b]
Lecithinase	Not tested					+	0	0	0	0				
Salicin (acid)	+	0	0	0	0	Not tested					0	0	+	V
Esculin hydrolysis	+	0	0	0	0	Not tested					0	0	+	V
Indole production	+	+	0	0	0	+	+(L)	0	0	0	0	+	+	V
Xylose (acid)[c]	+	+	+	0	0	+	+	+	0	0	V	+	+	V
Lactose (acid, of medium)[c]	+	+	+	0	0	+	+	+	0	0				
Nitrate reduction	+	+	+	+	0	+	+	+	+	0				
Trehalose (acid)	+	+	+	+	0	+	+	+	+	0				
Sorbitol (acid)	+	+	+	+	0	Not tested								
Ornithine decarboxylase[c]	+	+	+	+	0	+	+	+	+	0				
Voges-Proskauer[c]	+	+	+	+	0	Not tested								
β-Galactosidase[c]	+	+	+	+	0	+	+	+	+	0				
Sucrose[c]	+	+	+	+	0	Not tested								
Sorbose	+	+	+	+	0	Not tested								

Note: OF, oxidation-fermentation; +, positive; 0, negative; V, variable.

[a]Strains in Group 3 may produce gas in glucose.

[b]Strains in Group 4 may show atypical reactions; i.e. lactose, rhamnose positive.

[c]Test performed at 25°C.

Adapted from Bottone, 1977.

Wauters, using lecithinase activity as an additional characteristic and omitting some substrates utilized by Niléhn, devised a similar classification scheme in which isolates were divided into 5 biotypes.

On the other hand, Knapp and Thal (1973) separated *Y. enterocolitica* and *Y. enterocolitica*-like organisms into four groups on the basis of production of indole and of acid in xylose, esculin and salicin. Strains belonging to the first group show typical *Y. enterocolitica* reactions; strains of the second group represent *Y. enterocolitica* isolates that are indole-positive, while strains of Groups 3 and 4 are biochemically atypical.

Serologically, there are 34 "O" thermostable antigens and 19 "H" antigens recognized (Winblad, 1967; Wauters *et al.*, 1972. The former author established an antigenic schema with 9 serogroups; this was followed by Wauters' schema of 17 serogroups and a supplemental schema of 34 serogroups. Recently, Knapp and Thal (1973) proposed a simplified schema containing 6 O-groups indicating that further o-groups and subgroups will be proposed in the future.

A phage typing system has been developed in Europe by Nicolle *et al.* (1972) at the Pasteur Institute in Paris. He isolated strains of *Y. enterocolitica* phages which allow grouping of *Y. enterocolitica* strains into 9 main phage types; type 10 consists of strains not typable by phage. Most of the isolates from the United States are insensitive to the European typing bacteriophages (Lafleur *et al.*, 1972). This typing system has been useful in separating Canadian and European serotype 3; the former belonging to a "specific Canadian" type, 9b, and the latter to either phage type 8 or 9a.

The mechanism of pathogenicity of this organism has not been elucidated. Early investigation by Mollaret and Guillon (1965) resulted in no appropriate animal model being established since *Y. enterocolitica* isolates were described as non-pathogenic when introduced by the intraperitoneal, intravenous or subcutaneous routes into fifteen species of laboratory animals. However, Carter *et al.* (1974), Quan *et al.* (1974) and Alonso *et al.* (1975) have recently confirmed and extended animal pathogenicity studies first conducted and recorded by Schleifstein and Coleman in 1939.

Carter and Collins in their paper, described a clinical isolate highly virulent for mice by the intravenous and oral routes producing infection similar to the naturally acquired infection in man and that induced in mice. Quan and his co-workers reported killing of mice by two human isolates of *Y. enterocolitica* and one isolate of flea origin. Similarly, Alonso *et al.* have shown athymic mice to be of value for pathogenicity testing.

In describing experimental infections in mice caused by *Y. enterocolitica* isolates, investigators have improved our knowledge and insight into the factors contributing to the mechanism of human infection. However, such experiments have not fully elucidated the pathogenic mechanism of this organism (Bottone, 1977). Obviously, continuous monitoring in this area of research will be required to confirm and validate the pathogenicity of *Y. enterocolitica* isolates.

The clinical manifestations caused by *Y. enterocolitica* are very diverse and are dependent upon the age and physical state of the host. Several of the presentations such as acute mesenteric lymphadenitis and

terminal ileitis are seen mainly in older children and young adults, while typhoid-like septicemia usually occur in the aged, debilitated or patients on immunosuppressants (Hässig *et al.*, 1949; Daniels *et al.*, 1965; Toma, 1973; Keet, 1974; Rabson *et al.*, 1975). Erythema nodosum and arthritis are observed mainly in adults (Winblad, 1969; Jacobs, 1975; Winblad, 1975); while symptoms suggestive of enterocolitis are seen mostly in children (Schleifstein, 1943; Lafleur *et al.*, 1972; Toma *et al.*, 1972; Delorme *et al.*, 1974). Other manifestations include myocarditis (Ahvonen, 1972), subacute localized abscesses of liver and spleen (Rabson *et al.*, 1972; 1975), meningitis and panophthalmitis (Sonnenwirth, 1968), cellulitis (Abramovitch *et al.*, 1973; Rabson *et al.*, 1975), erysipelas-like eruptions (Hayen *et al.*, 1974); Reiter's disease (Solem and Lassen, 1971; Aho *et al.*, 1974); hemolytic anemia (Von Knorring and Pettersson, 1972) and a furuncle caused by *Y. enterocolitica* (Lawrence *et al.*, 1975).

The foregoing indicates that human infection with *Y. enterocolitica* has several clinical manifestations, thus it would be worthwhile to emphasize the more important characteristics associated with the clinical syndromes most frequently encountered in yersiniosis.

ENTERITIS

Enteritis was first observed as early as 1939 by Schleifstein and Coleman in the U.S. who first described and recognized this infection caused by *Y. enterocolitica*. It is the most frequently encountered manifestation of yersiniosis and is considered by most researchers to comprise at least two-thirds of all reported cases (Mollaret, 1972). This disease, which occurs primarily in infants and young children, is characterized by diarrhea which may last from two to several weeks,

occasionally accompanied by blood and mucus in the stool (Zen-Yoji *et al.*, 1973 and Bergstrand and Winblad, 1974). Vomiting may be present, but is usually not prominent. Severe abdominal pain in the right lower quadrant, highly suggestive of acute appendicitis, may also be encountered - a finding usually followed by surgery, particularly in children and young adults between the ages of 10 and 19 years of age.

Infection caused by *Y. enterocolitica* produces clinical symptoms almost indistinguishable from those caused by other enteropathogens (*Salmonella*, *Shigella* and enteropathogenic *E. coli*) (Rabson *et al.*, 1972; Vandepitte *et al.*, 1973; Delorme *et al.*, 1974; and Schieven and Randall, 1974). Hence, diagnosis depends solely upon recovery of this micro-organism from enteric contents during the acute enteric phase. Usually, *Y. enterocolitica* Serotype 3, Biotype 4 and Serotype 9, Biotype 2, and less frequently, Serotype 8, have been associated with enteritis when this organism was isolated and further identified in clinical laboratories.

The incidence of *Y. enterocolitica* enteritis is reported by some specialists to have surpassed the incidence of *Shigella*, being next, only to *Salmonella* (Toma, 1973). Cases of enteritis caused by this organism reported within the last decade have been either sporadic or confined to small family outbreaks. Such reports were described in Canada (Toma *et al.*, 1972); Japan (Zen-Yoji, 1973), Finland (Ahvonen, 1973) and in the U.S.A. (Gutman *et al.*, 1973; and Black *et al.*, 1978).

ACUTE MESENTERIC LYMPHADENITIS AND TERMINAL ILEITIS

These two closely associated diseases were first described in 1953 by Masshoff in children who demonstrated symptoms clinically indistinguishable from acute appendicitis. Normally, patients suffering from

these *Y. enterocolitica* infections experience acute pain in the lower right quadrant section of the abdomen simulating acute appendicitis. At surgery, in most cases, the appendix is found to be grossly normal or only slightly inflamed in appearance. However, enlarged lymph nodes are usually observed. Saebo (1974) in his paper "Some surgical manifestations of mesenterial lymphadenitis associated with infection of the *Yersinia enterocolitica*" reported the observation of extensive swelling of the mesenteric lymph nodes to the extent of mimicking a tumor. Pathologically, inflammation is restricted to the terminal ileum and cecal section of the intestines, but histologically, the lymph nodes indicate proliferation of large pyroninophilic cells with large nucleoli in the cortical region of the pulp (Ahlqvist *et al.*, 1971).

ARTHRITIS

The association of *Y. enterocolitica* infection and arthritis was first described in Finland by Ahvonen and his co-workers in 1969. They found that it was almost always associated with yersiniosis in young adults and rarely in infants, and that the symptoms were manifested mainly in the joints of the knees, ankles and fingers. Following this report numerous other researchers (Laitinen *et al.*, 1972; Aho *et al.*, 1974; Jacobs, 1975 and Winblad, 1975) have published papers associating post infectious arthritis with *Y. enterocolitica* infections.

In European countries, patients demonstrating post infectious arthritis are usually previously exposed to infection with *Y. enterocolitica* Serotype 3 or 9. In Canada, *Y. enterocolitica* Serotype 3, Biotype 4 is the most common strain recovered, however arthritis following yersiniosis have not been observed in patients (Toma and

Lafleur, 1974). In South Africa, *Y. enterocolitica* infections were first described in 1971 by Rabson *et al.*, but the picture is the same as that observed in Canada with respect to post infectious arthritis associated with *Y. enterocolitica*.

ERYTHEMA NODOSUM

Erythema nodosum was first described by Mollaret and Destombes (1964) in a female patient following an enteric infection with *Y. enterocolitica*. This patient was described as developing a typical erythema nodosum 17 days after surgery which revealed mesenteric lymphadenitis. Erythema nodosum manifests itself usually on the lower extremities of the patient, followed by the arms, and, on occasion, the upper trunk (Arvastson *et al.*, 1971).

SEPTICEMIA

Septicemia due to *Y. enterocolitica* is a disease now receiving undue attention since a 50% mortality rate has been reported in patients suffering from this condition (Mollaret *et al.*, 1971). It has been observed primarily in the aged and immune-deficient or immune-suppressed patients whose host defenses have been lowered (Winblad, 1973).

This disease may have a rapid onset and is usually accompanied by high fever, general malaise, vomiting and diarrhea. This is the acute septicemic manifestation mimicking typhoid fever or malaria, as described by Rabson *et al.* in 1975. Another clinical presentation described by these workers, is the subacute localizing form, resembling amebic hepatitis, in which a history of recent diarrhea and vomiting is usually absent.

ANTIBIOTIC SUSCEPTIBILITY

Presently, there is no single antibiotic regime that could be

adopted for the treatment of *Y. enterocolitica* infection. In 1974 in Ontario, Canada, Toma performed susceptibility tests on stock cultures and *Y. enterocolitica* isolates and found that they were highly susceptible to the tetracyclines (except two cultures), amino-glycosides (gentamicin, kanamycin, streptomycin), chloramphenicol, nalidixic acid, colistin and sulfadiazine (except three cultures). Gutman *et al.* (1973) further found the combination of trimethoprim and sulfamethoxazole to be highly effective against *Y. enterocolitica.* Resistance to penicillin, oxacillin, lincomycin and novobiocin have been reported by Toma. Partial resistance to ampicillin and erythromycin with moderate to little resistance or susceptibility to cephaloridine and carbenicillin was also recorded.

The administering of antiobiotics in cases of *Y. enterocolitica* infection should be done with reservations. Antibiotic therapy is not recommended in enteritis since this infection is usually mild and self-limiting. However, in more severe infections where *Y. enterocolitica* may become systemic, antibiotic therapy (chloramphenicol and colistin) is highly recommended since extreme cases of yersiniosis may develop if left untreated.

Efforts to reveal the mode of transmission of *Y. enterocolitica* from its natural reservoir to produce human infections have, to date, been futile. Although there are numerous reports describing both single cases of enteritis and large outbreaks involving several members in various group situations, only one of these cases has been definitely traced back to a specific source. In this outbreak, the drinking of contaminated chocolate milk was epidemiologically associated with

Y. enterocolitica infection (Black *et al.*, 1978). Person to person transmission of *Y. enterocolitica* from ill children to household contacts did not occur in this reported outbreak, a finding similar to that in three Japanese school outbreaks (Asakawa *et al.*, 1973; Zen-Yoji *et al.*, 1973) but in contrast to two other outbreaks in which person to person transmission was suggested (Toivanen *et al.*, 1973; Gutman *et al.*, 1973).

Recent reports indicate that the animal kingdom serves as a major reservoir for *Y. enterocolitica* (Toma and Deidrick, 1975; Kapperud and Jonsson, 1976). However, strains recovered from rodents (Kapperud, 1975), hares (Mollaret *et al.*, 1965), chinchillas (Hubbert, 1972; Vandepitte *et al.*, 1973) and water sources (Lassen, 1972) are usually a bioserotype not associated with human disease. On the other hand, *Y. enterocolitica* strains of serotype and phage type identical to those recovered from human hosts have been isolated from animals, particularly swine (Tsubokura *et al.*, 1973; Zen-Yoji *et al.*, 1974; Toma and Deidrick, 1975). Furthermore, investigators in Europe (Mollaret, 1971), Japan (Tsubokura *et al.*, 1973; Zen-Yoji *et al.*, 1974), South Africa (Rabson and Koornhof, 1972) and Canada (Toma, 1973; Toma *et al.*, 1975) have all demonstrated that swine serve as the major reservoir for *Y. enterocolitica* Serotype 3 strains ultimately encountered in humans. Serotype 9, isolated in Europe and Canada only, and thought to be carried and transmitted solely by man, has also been recovered from the pig (Esseveld and Goudzwaard, 1973) and from a dog and a cat with symptoms of enteritis (Ahvonen *et al.*, 1973). Serotype 8 - encountered predominantly in the U.S. and also isolated in the Western areas of Canada (Toma, 1974) has been isolated from a variety of mammals (Hubbert, 1972). Keet (1974), in his

report of septicemia due to Serotype 8 *Y. enterocolitica* in man, has suggested the possibility of the deer serving as a reservoir for this serotype in the U.S.

Based purely on circumstantial evidence, it would appear that this organism can also be spread via direct or indirect human transmission. A number of reports (Albert and Lafleur, 1971; Zen-Yoji and Maruyama, 1972; Bergstrand and Winblad, 1974; Delorme *et al.*, 1974) present evidence in which *Y. enterocolitica* infections occurred in young children who have not had animal contact. Further reports involving familial and interfamilial outbreaks (Ahvonen and Rossi, 1970; Gutman *et al.*, 1973) and sporadic outbreaks (Toivanen *et al.*, 1973; Zen-Yoji *et al.*, 1973; Olsovsky *et al.*, 1975) also support the interhuman mode of transmission.

Very little work has been carried out on methods of controlling the spread of this disease. This area of knowledge cannot be developed further until (1) the means through which *Y. enterocolitica* is spread is understood and (2) its reservoirs are known. This organism is widespread in nature in both living and non-living systems. Therefore general techniques of environmental hygiene and sanitation with regard to food and water should apply in controlling disease caused by *Y. enterocolitica* (Morris and Feeley, 1976).

Yersinia enterocolitica can be truly described as a uniquely elusive microorganism since factors contributing to (1) the geographic distribution and localization of certain serotypes, (2) its mode of transmission, (3) its reservoirs and (4) pathogenicity still remain to be resolved, irrespective of the vast number of years that European, Canadian and American researchers have been devoting their efforts to

the un-covering of this microorganism.

A review of the literature showed that information on the growth temperature relationship of *Yersinia enterocolitica* was limited. The main objective of this investigation was to establish definitive information on the temperature response of this organism in complex medium, minimal medium, and meat. In addition the effects of sodium chloride and pH were to be studied. A secondary objective was to evaluate the use of enteric media inoculated at refrigerated temperatures for isolation of *Y. enterocolitica*. If successful, this method could be a significant addition to current methodology for isolation and differentiation of members of the family *Enterobacteriaceae*.

MATERIALS AND METHODS

A. *TEMPERATURE STUDIES*

Test Organism and Growth Media

Three cultures of *Y. enterocolitica* were obtained from the Department of Medical Bacteriology, University of Alberta, Edmonton, Alberta, Canada. One culture originated from the American Type Culture Collection (ATCC 23715), Rockville, Maryland, USA and the other two were clinical isolates, designated Serotype 0:3 and Serotype 0:9. The latter were used in the study because they have been reported to be the predominant human serotypes isolated from Europe and Canada (Toma and Lafleur, 1974). Each of the test cultures was maintained as a stock on Trypticase Soy Agar (TSA) slants stored at 4°C. At monthly intervals, a loopful of the stocks was transferred to fresh TSA slants, incubated at 37°C for 48 hrs, and then returned to storage at 4°C. Purity of cultures was examined by Gram staining at each monthly subculture.

Trypticase Soy Broth (TSB, Baltimore Biological Laboratory [BBL]) constituted the complex medium and Glucose Salts Medium (GSM) was the minimal broth employed. The formula for GSM was adapted from "Microorganisms in Food 1", Thatcher and Clark (1975):

Tryptone 2g

NaCl 5g

Potassium monohydrogen phosphate 0.3g

Ingredients were dissolved in 900 mls distilled H_2O. 10g glucose was made up to 100 mls in distilled H_2O, sterilized separately and mixed to the sterilized salt solution after cooling.

pH of the growth medium was adjusted using hydrochloric acid before sterilization so that the desired pH was obtained after sterilization. The sodium chloride (NaCl) content of the medium was adjusted by the addition of appropriate quantities before sterilization.

All media were prepared and sterilized as stipulated by the manufacturers.

Preparation of Inoculum

A 250 ml Erlenmeyer flask containing 100 ml aliquots of growth medium (TSB or GSM) was inoculated with a loopful of the test organism from the stock agar slant. This was incubated at 37°C in a Gyratory Shaker (New Brunswick Scientific Company, New Brunswick, N.J.) operated at 150 rpm. An aliquot from this culture at the mid-stationary phase (determined by a growth curve experiment) was transferred to a new pretempered flask containing 100 ml growth medium. Three such subcultures were performed, the final subculture constituting the inoculum. A 1% inoculum was used constantly throughout the study.

Growth Response

Growth response in relation to incubation temperature was determined in two ways. A Temperature Gradient Incubator (Model #TGI, Scientific Industries Inc., N.Y.) was used for temperatures in the range 10°-50°C. The Temperature Gradient Incubator (TGI) was set to give the desired temperature range. Special L-shaped culture tubes containing 15 ml of growth medium were placed in the holes in the TGI and allowed to equilibrate overnight. The temperature of each tube was determined with a thermocouple inserted into a duplicate set of tubes. Temperatures were monitored and recorded throughout the duration

of the experiment. The pretempered culture tubes were inoculated with 0.15 ml of the prepared test culture and agitated at 45 strokes/min.

Temperature relationships in the range 0°-8°C were determined from experiments using 2 ℓ Erlenmeyer flasks. One litre aliquots of growth medium were dispensed into the flasks together with a Teflon coated magnetic stirring bar. After sterilization the flasks were placed in 12" x 12" x 12" perspex tanks into which water of the desired temperature was circulated from temperature control units (Precision Scientific, Chicago, Illinois). The stirring bar was activated by placing the perspex tank and culture flask on a non-heating magnetic stirrer (Bellco Glass Inc., Vineland, N.J.). The flasks were allowed to equilibrate for 24 h prior to inoculation.

Growth was determined by measurement of absorbance (600 nm) at hourly intervals for the first twelve hours and subsequently at 24 hr intervals, using a Spectronic 20 (Bausch and Lomb, Rochester, New York) and by viable colony count. Viable counts were made by surface plating appropriate decimal dilutions on prepared predried TSA plates. The plates were incubated at 37°C for 48 hr and then counted. The results were expressed as colony forming units (c.f.u.). All dilutions were made in 0.1% Bacto-peptone, pH 7.2.

B. *SELECTIVE ISOLATION STUDIES*

Test Organisms and Growth Media

Members of the family *Enterobacteriaceae* were used in this investigation. These included the *Y. enterocolitica* cultures used in the previous study, and different species of the following: *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Serratia*, *Arizona*, *Enterobacter*, *Citrobacter*, *Escherichia* and *Providencia* as well as psychrophilic

Pseudomonas sp.

All cultures were isolated and biochemically identified at the Provincial Laboratory of Public Health, Edmonton, Alberta, Canada. Stock cultures were maintained at 4°C on Trypticase Soy Agar, subcultured and Gram-stained monthly.

Enteric media investigated were: Bismuth Sulfite (BS), Brilliant Green (BG), Deoxycholate Citrate (DC), Eosin Methylene Blue (EMB), Endo, MacConkey, Salmonella-Shigella (SS), Violet Red Bile (VRB) and Violet Red Bile Glucose (VRBG). The non-selective, complex medium, Trypticase Soy Agar (TSA, BBL) was included in this study for comparative purposes.

All enteric media were obtained from Difco Laboratories Ltd., Detroit, Michigan, USA, and were prepared as recommended by the manufacturer.

Preparation of Inoculum

A 10 ml aliquot of Trypticase Soy Broth (TSB) was inoculated with a loopful of the test organism from the stock agar slant. This was incubated in a hot air incubator at 37°C for 16 hrs. An aliquot from this culture at the mid-stationary phase (determined by a growth curve experiment) was transferred to a sterile tube of pretempered TSB. Two such subcultures were made, the final subculture representing the inoculum. Inoculum for all test cultures used in the experiment was prepared as described above.

Viable Counts

Dilutions of the TSB cultures were prepared by transferring 1.0 ml aliquots to 9.0 ml sterile peptone water blanks (0.1% Bacto-peptone, pH 7.2). Dilutions were vortexed for fifteen seconds and ten-fold serial

dilutions (10^{-1} to 10^{-7}) were prepared. 0.1 ml aliquots of the appropriate decimal dilutions were then surface-plated, in duplicate, on pre-dried TSA plates (inoculum count), and on the appropriate selective media. TSA plates were incubated at 37°C for 48 hrs. Colonies formed after incubation were counted with the aid of a dark field colony counter and the number of colony forming units (c.f.u.) in the original sample was calculated.

During preliminary testing and screening of temperatures, enteric media plates were streaked with a loopful of undiluted inoculum (16 hrs growth culture) in order to obtain isolated colonies. In subsequent quantitative studies, enteric plates were inoculated with the appropriate decimal dilutions of inoculum, which was surface-plated, and incubated at test temperatures until colonies were observed. The percentage (%) recovery rate was calculated.

C. *PORK INOCULATION STUDIES*

Test Organism and Inoculum Preparation

Yersinia enterocolitica Serotype O:3 was selected as the test organism because reports indicate that pigs serve as the major reservoir for this Serotype ultimately encountered in humans (Mollaret *et al.*, 1971; Toma, 1973). The inoculum, grown in TSB, was prepared as previously described in "Preparation of Inoculum"

Media

In the enumeration of total viable counts, TSA plates were used. *Y. enterocolitica* counts were obtained on VRBG and BS agars. VRBG agar was chosen because results obtained in the previous study indicated that it was the most suitable for isolating *Y. enterocolitica*. In a comprehensive report by Hanna *et al.* (1977) it was suggested that BS

agar be utilized when isolating *Y. enterocolitica* from meats, hence the feasibility of its use was also investigated in this study.

Meat Samples

Pork loin roast for inoculation was swabbed with ethanol and the sides sliced off with a sterile knife thus exposing a relatively aseptic centre portion. Samples (4 x 4 x 1/2 cm), weighing approximately 10g, were placed in sterile polythene bags and equilibrated at the test temperatures. Pork samples were then inoculated by spreading 0.1 ml inoculum over the upper surface. At each sampling interval, duplicate bags were removed from the incubator and viable counts were performed. Uninoculated controls, which were sampled at 0 hr and at the end of the experimental period, were also included in the study.

Viable Counts

Viable cell counts were determined by surface-plating in triplicate, 0.1 ml aliquots of the serially diluted homogenate on TSA, and incubating at 37°C for 48 hrs. The homogenate was prepared by "stomaching" the sample in 90 mls sterile 0.1% Bacto-peptone water for 3 mins.

Selective isolation of *Y. enterocolitica* was determined by surface-plating, in duplicate, 0.1 ml aliquots of the homogenate on VRBG and BS agars and incubated at 5°C for 10 days.

Bacteriological Identification

The distribution of microflora was determined by using a template and randomly picking 10 colonies from countable plates (i.e. plates containing 30-300 colonies) and inoculating them on TSA plates at 37°C for 48 hrs. Key biochemical tests were performed (Gram stain, catalase and oxidase) and *Y. enterocolitica* counts were confirmed by use of the

API 20E microtube system (Analytab Products, Plainview, N.Y.) on all Gram negative, oxidase negative rods. *Y. enterocolitica* counts from TSA plates were calculated from the total counts and the percentage of this organism among the isolates picked from countable plates.

RESULTS

The results of this study are most conveniently considered under three sections: 1) Temperature relationships of *Yersinia enterocolitica*, 2) Selective isolation at refrigerated temperature using enteric media, and 3) Development of *Y. enterocolitica* inoculated onto raw pork samples.

1. *TEMPERATURE RELATIONSHIPS OF Y. ENTEROCOLITICA*

A. Growth and Viability in TSB, pH 7.0

The growth of three strains of *Y. enterocolitica* in TSB (pH 7.0), as determined by absorbance, are presented in Figs. 1-6. The growth pattern of the three strains was comparatively similar. The optimum temperature for all strains is approximately 32°C, and the maximum growth temperature is 42°C. The minimum growth temperature for *Y. enterocolitica* ATCC 23715, and Serotypes 3 and 9 was found to be 0.5°, 1.4° and 1.2°C respectively after 7 days incubation. Serotype 3 grew at 0.6°C after a 12 day incubation period.

Growth rates of Serotype 3, expressed as generation time, are shown in Fig. 7.

The growth of the three strains of *Y. enterocolitica* as determined by viable counts, is shown in Figs. 8-13. Figs. 8, 9 and 10 do not represent true growth curves since they are based on only three points. However, they do indicate maximum population densities and trends in viability. At temperatures above 20°C population densities from 2×10^9 to 5×10^9 c.f.u./ml were achieved within 24 hours (Figs. 8, 9 and 10). Similar population densities were achieved at lower temperatures after more prolonged incubation (Figs. 11, 12 and 13). Loss of viability was

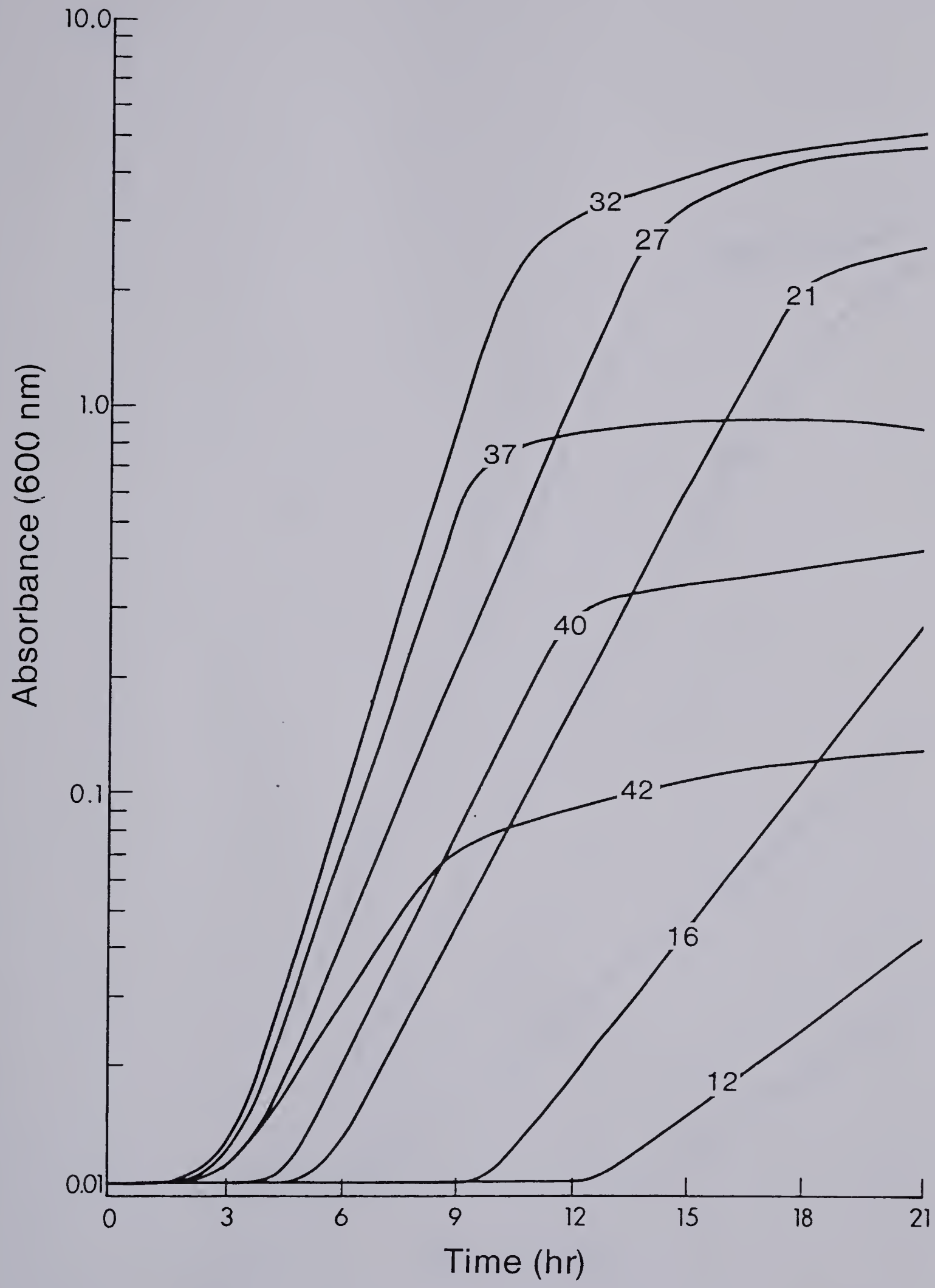


Fig. 1. Effect of temperature on growth of *Y. enterocolitica* ATCC 23715 in trypticase soy broth, pH 7.0.

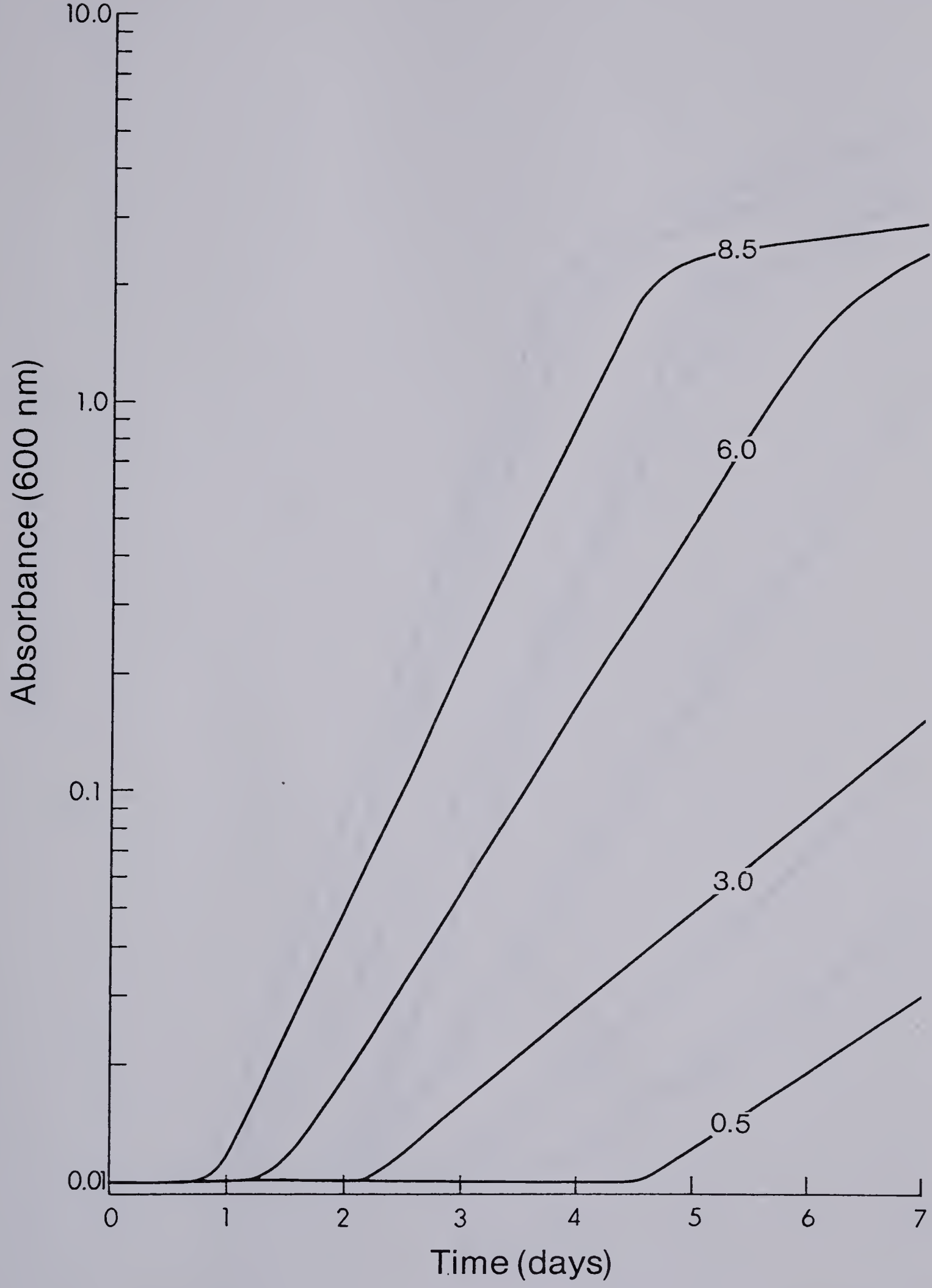


Fig. 2. Effect of temperature on growth of *Y. enterocolitica* ATCC 23715 in trypticase soy broth, pH 7.0.

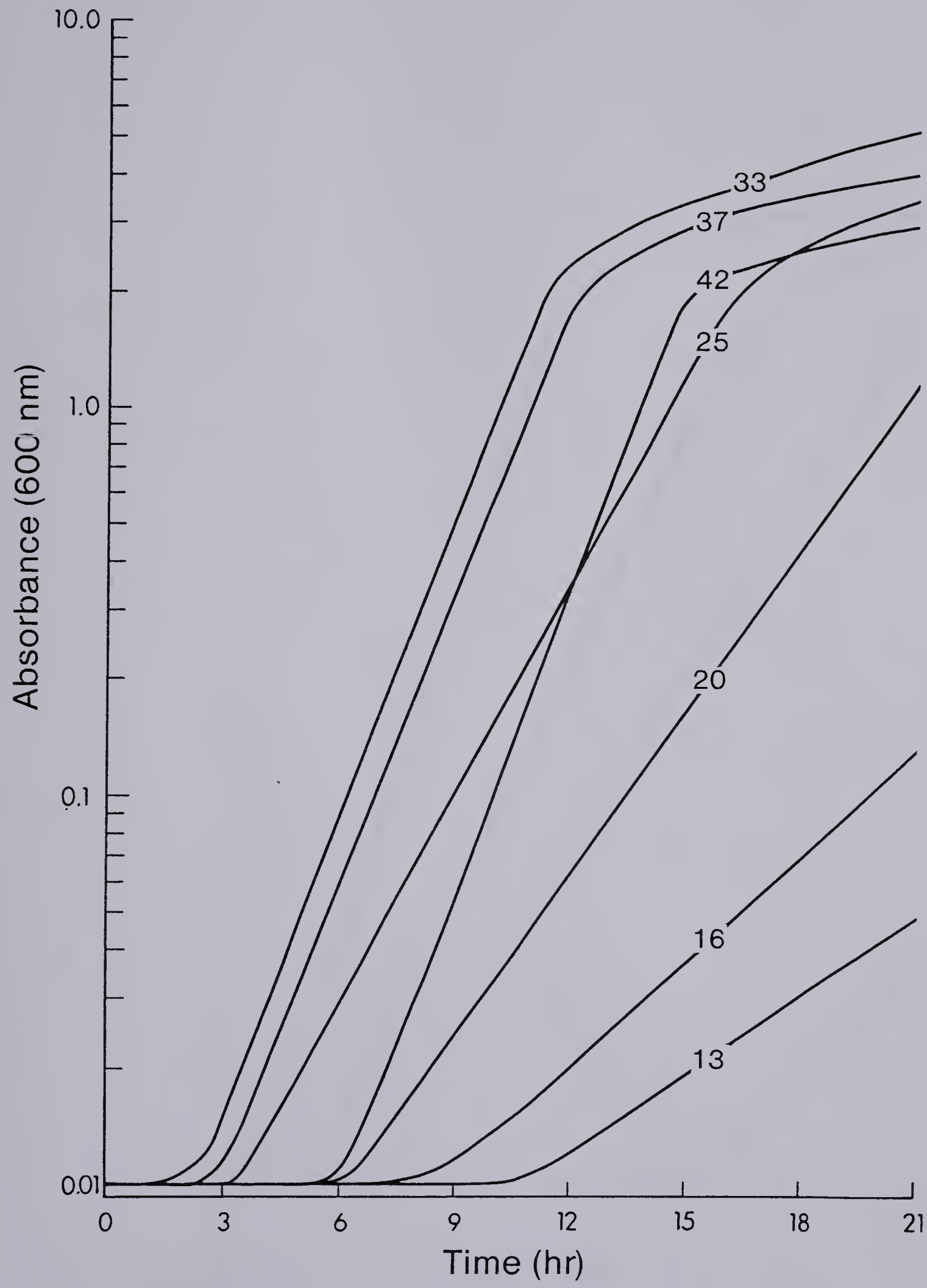


Fig. 3. Effect of temperature on growth of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 7.0.

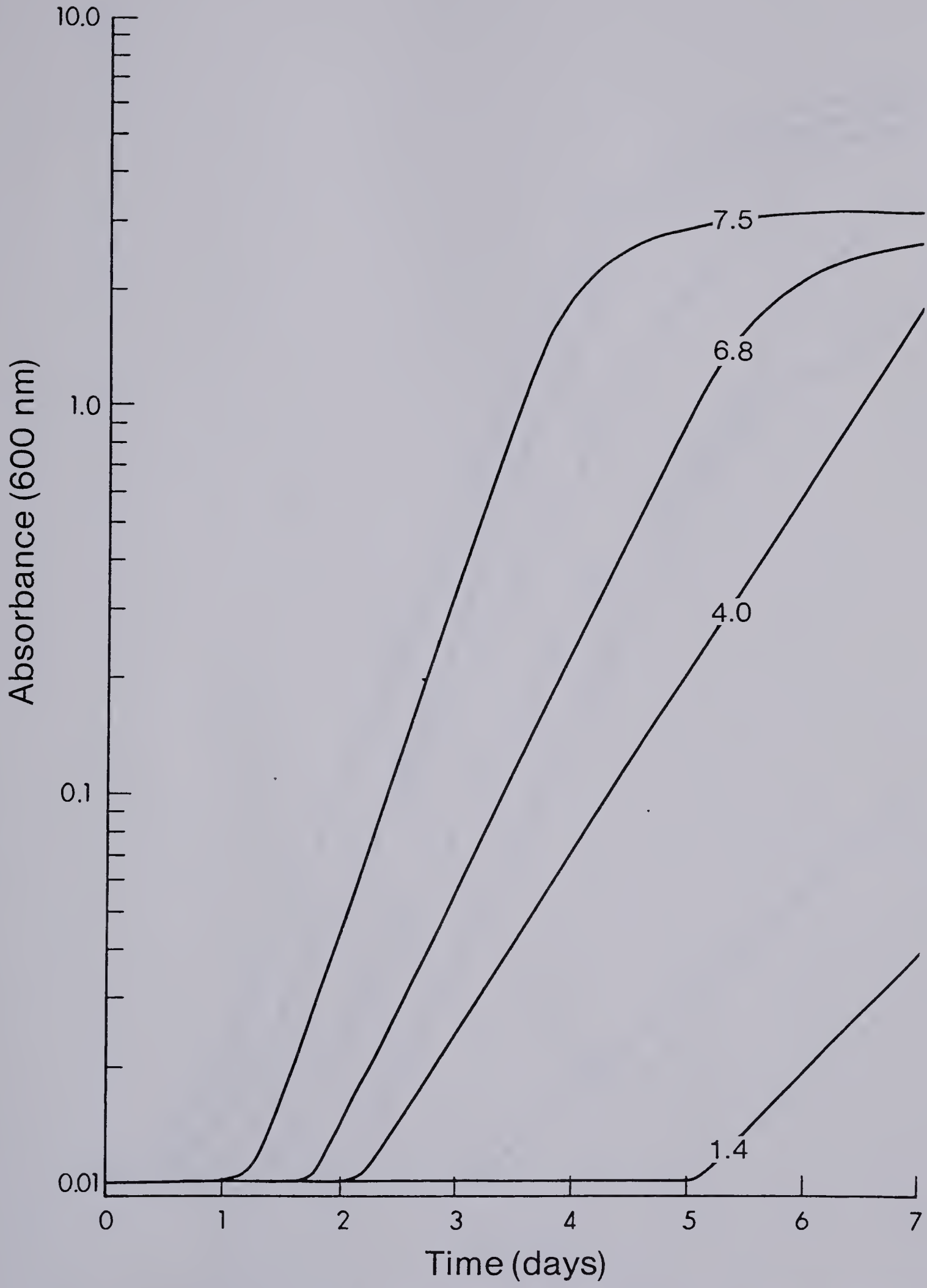


Fig. 4. Effect of temperature on growth of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 7.0.

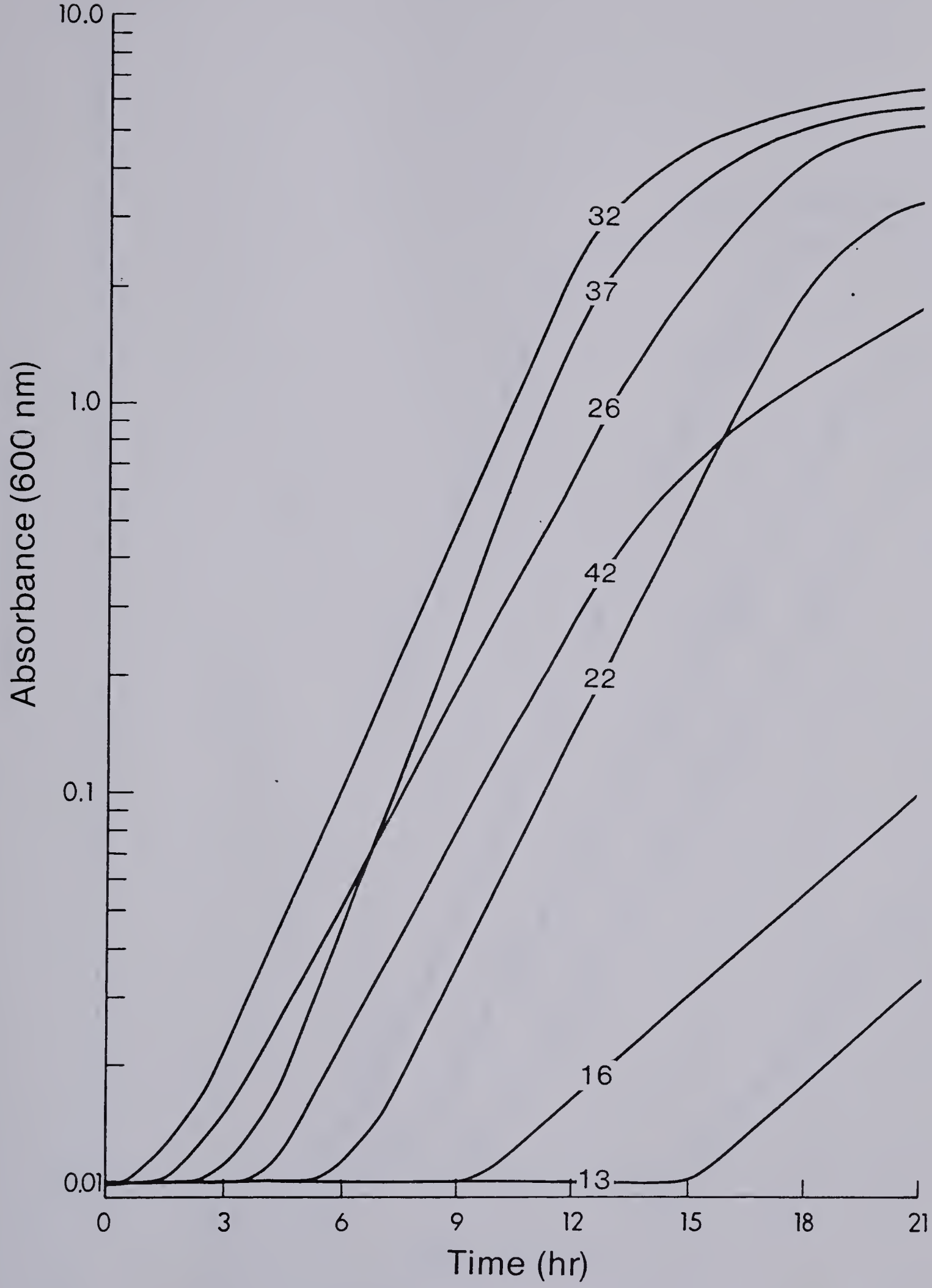


Fig. 5. Effect of temperature on growth of *Y. enterocolitica* Serotype 9 in trypticase soy broth, pH 7.0.

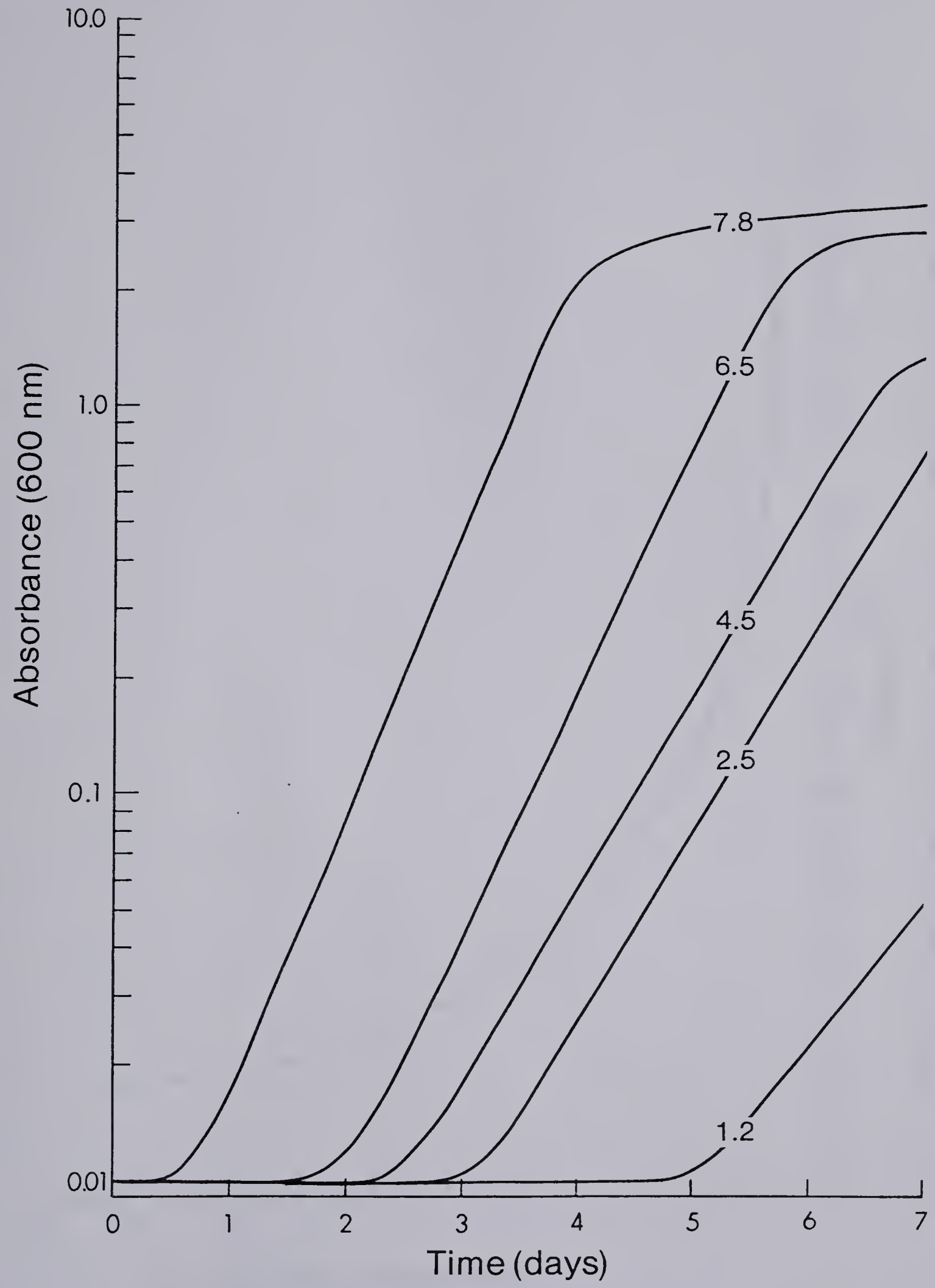


Fig. 6. Effect of temperature on growth of *Y. enterocolitica* Serotype 9 in trypticase soy broth, pH 7.0.

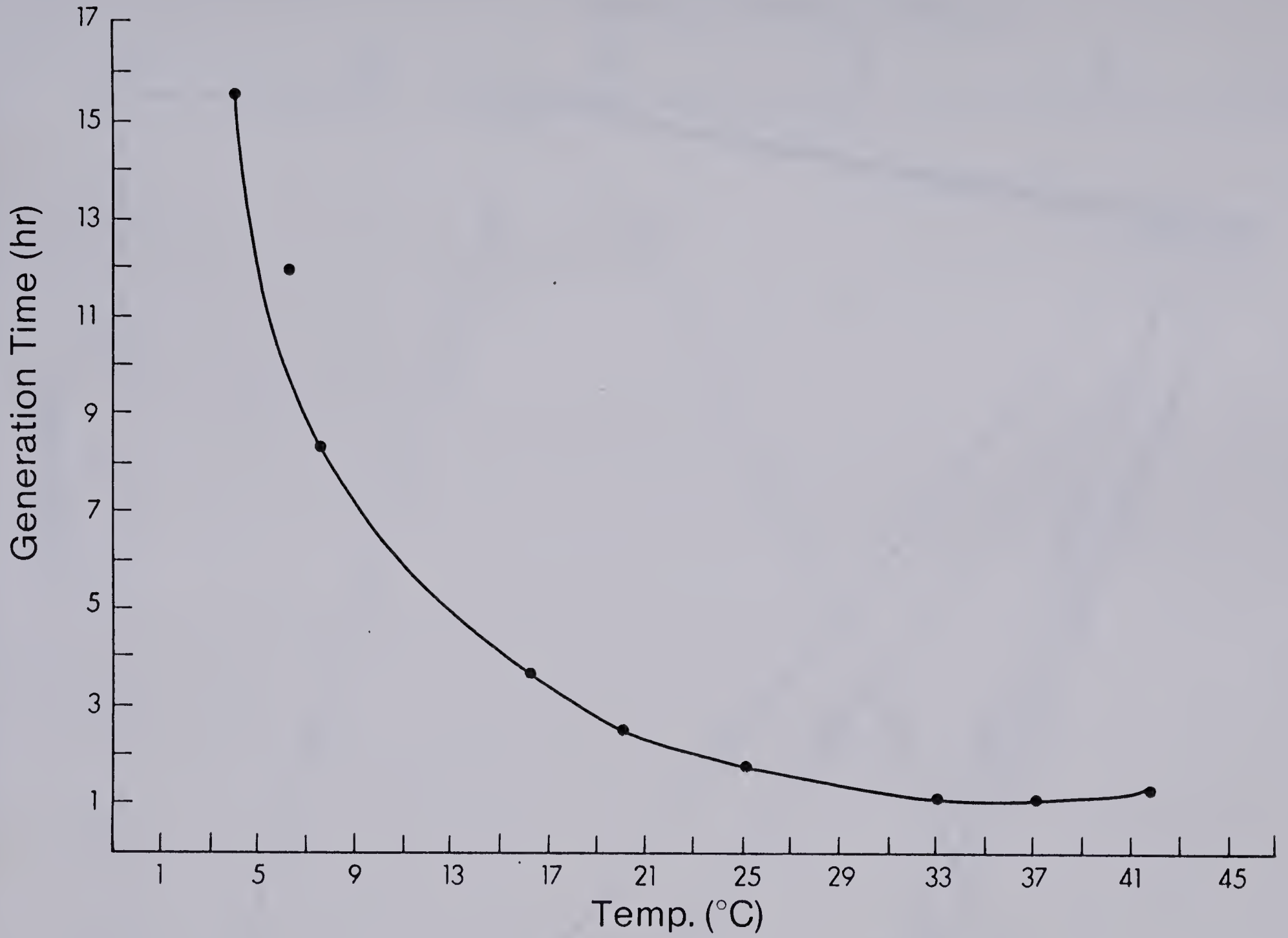


Fig. 7. Effect of temperature on generation time of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 7.0.

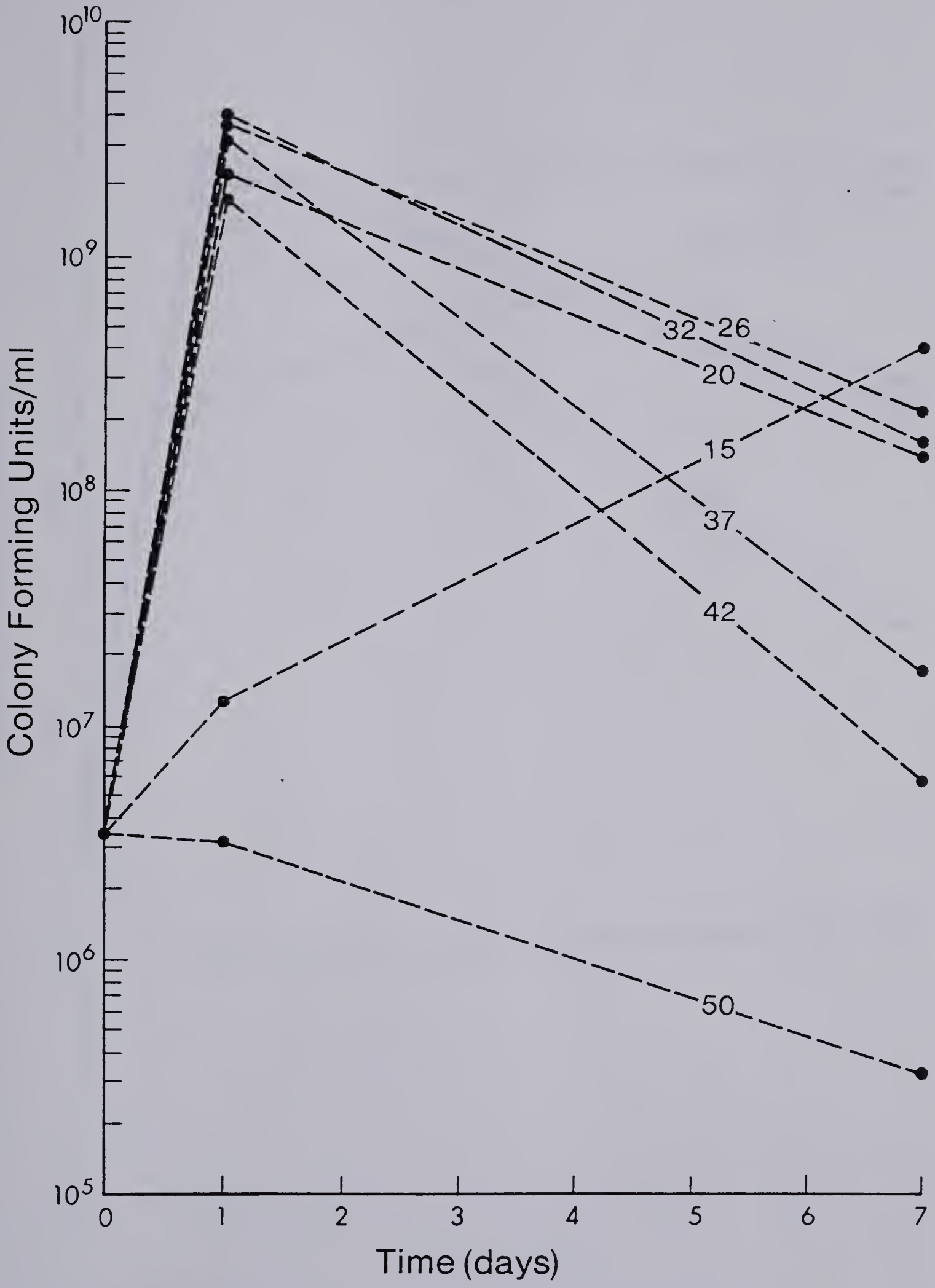


Fig. 8. Effect of temperature on growth and viability of *Y. enterocolitica* ATCC 23715 in trypticase soy broth, pH 7.0.

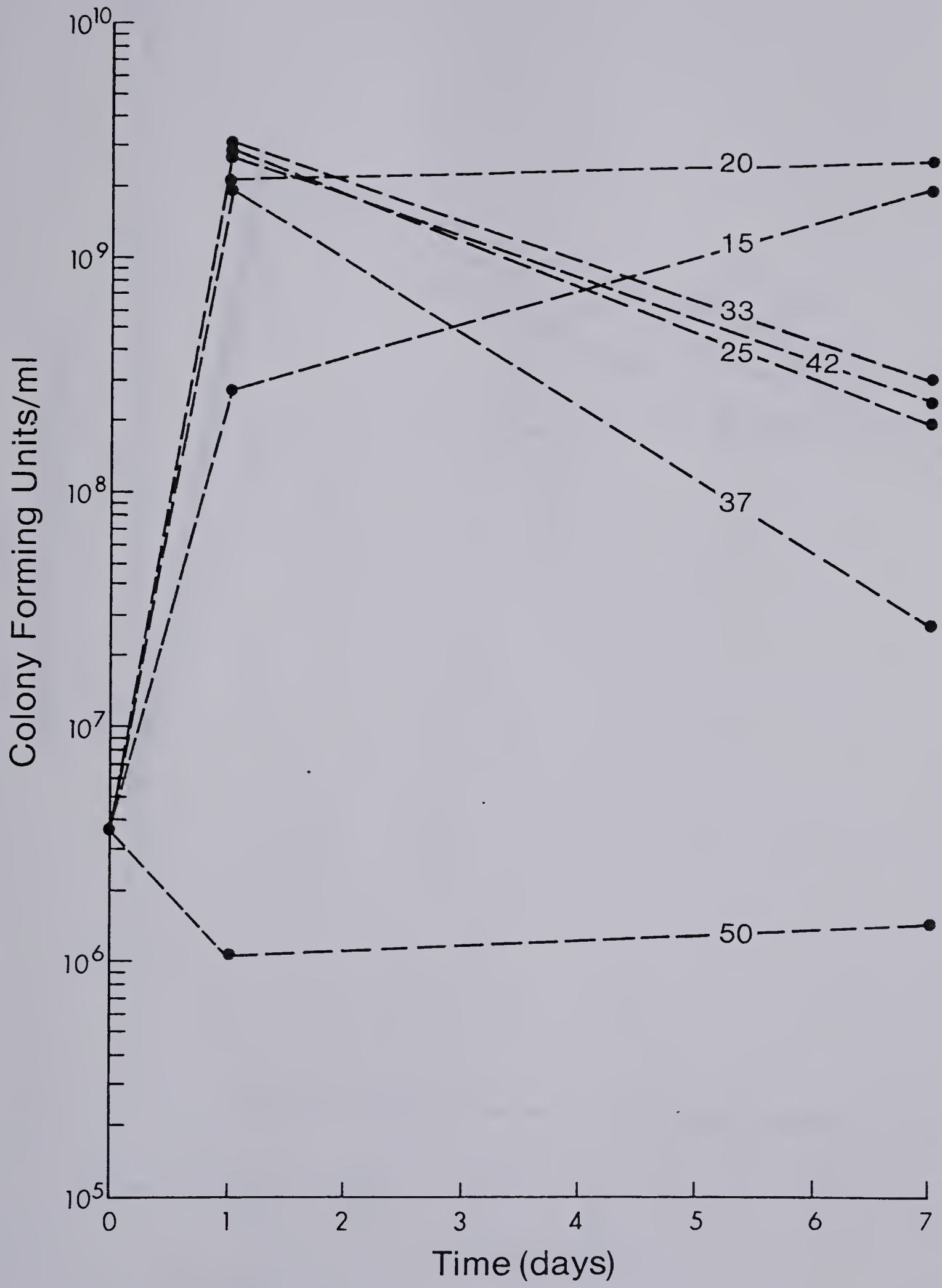


Fig. 9. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 7.0.

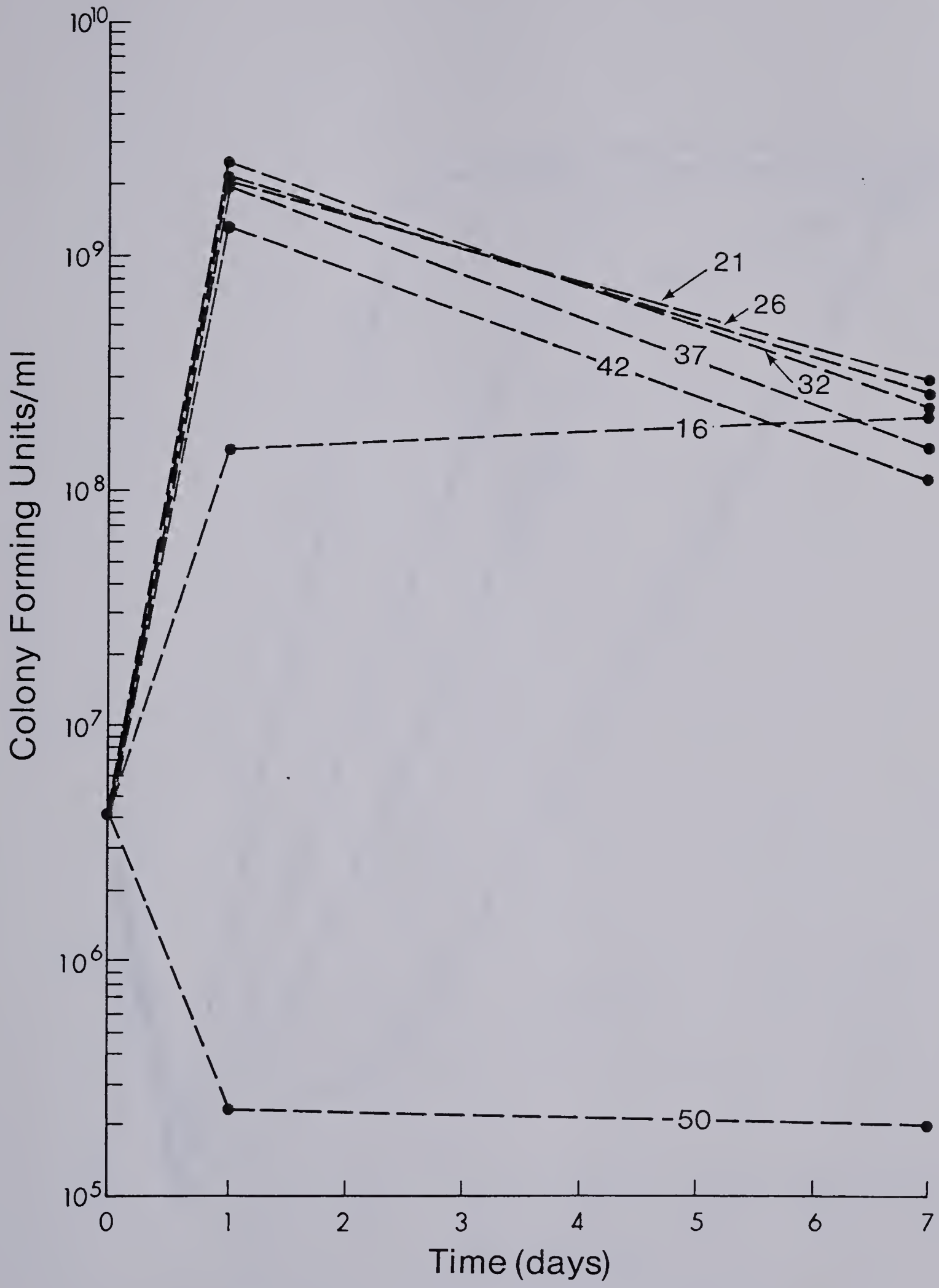


Fig. 10. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 9 in trypticase soy broth, pH 7.0.

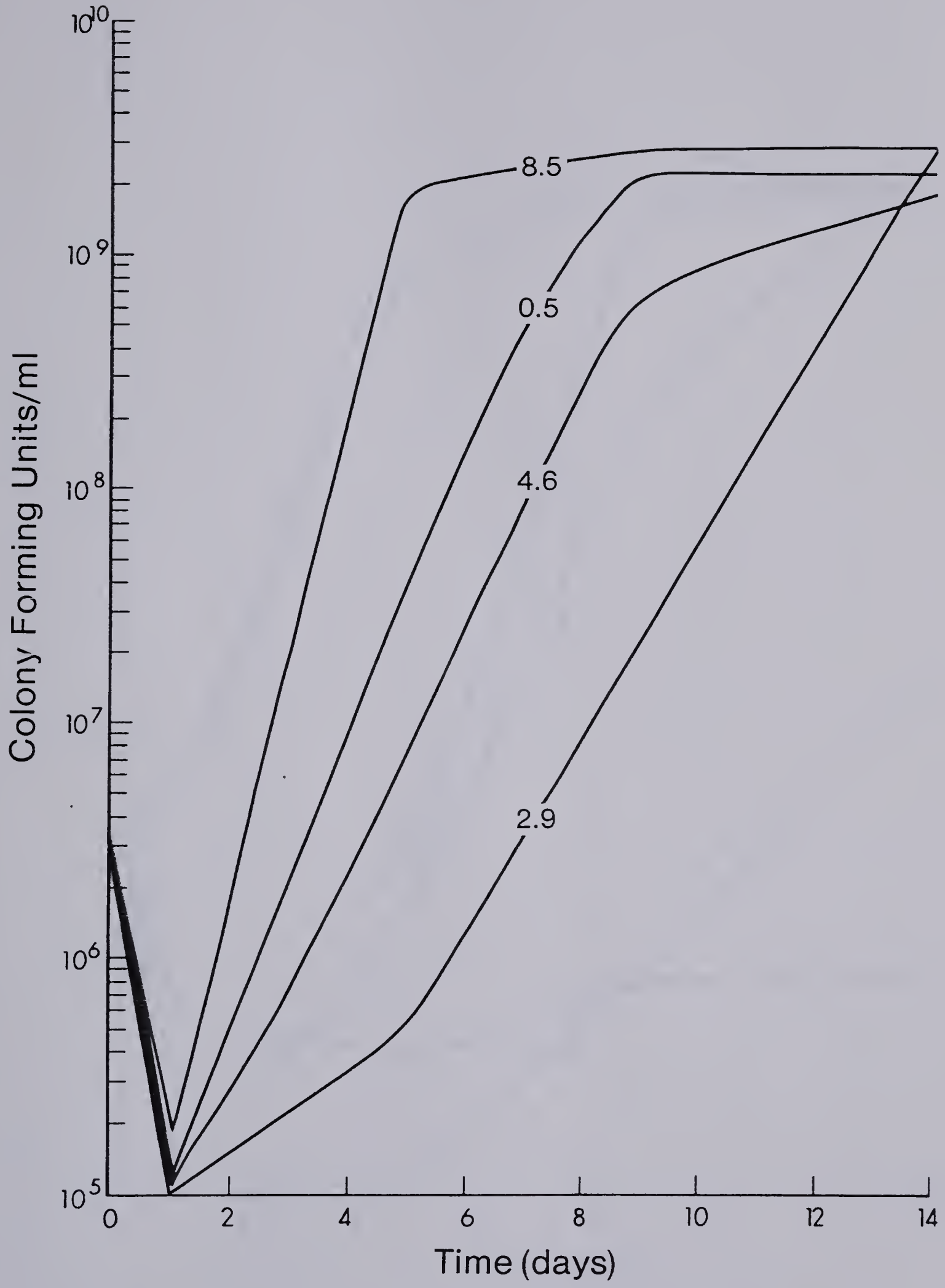


Fig. 11. Effect of temperature on growth and viability of *Y. enterocolitica* ATCC 23715 in trypticase soy broth, pH 7.0.

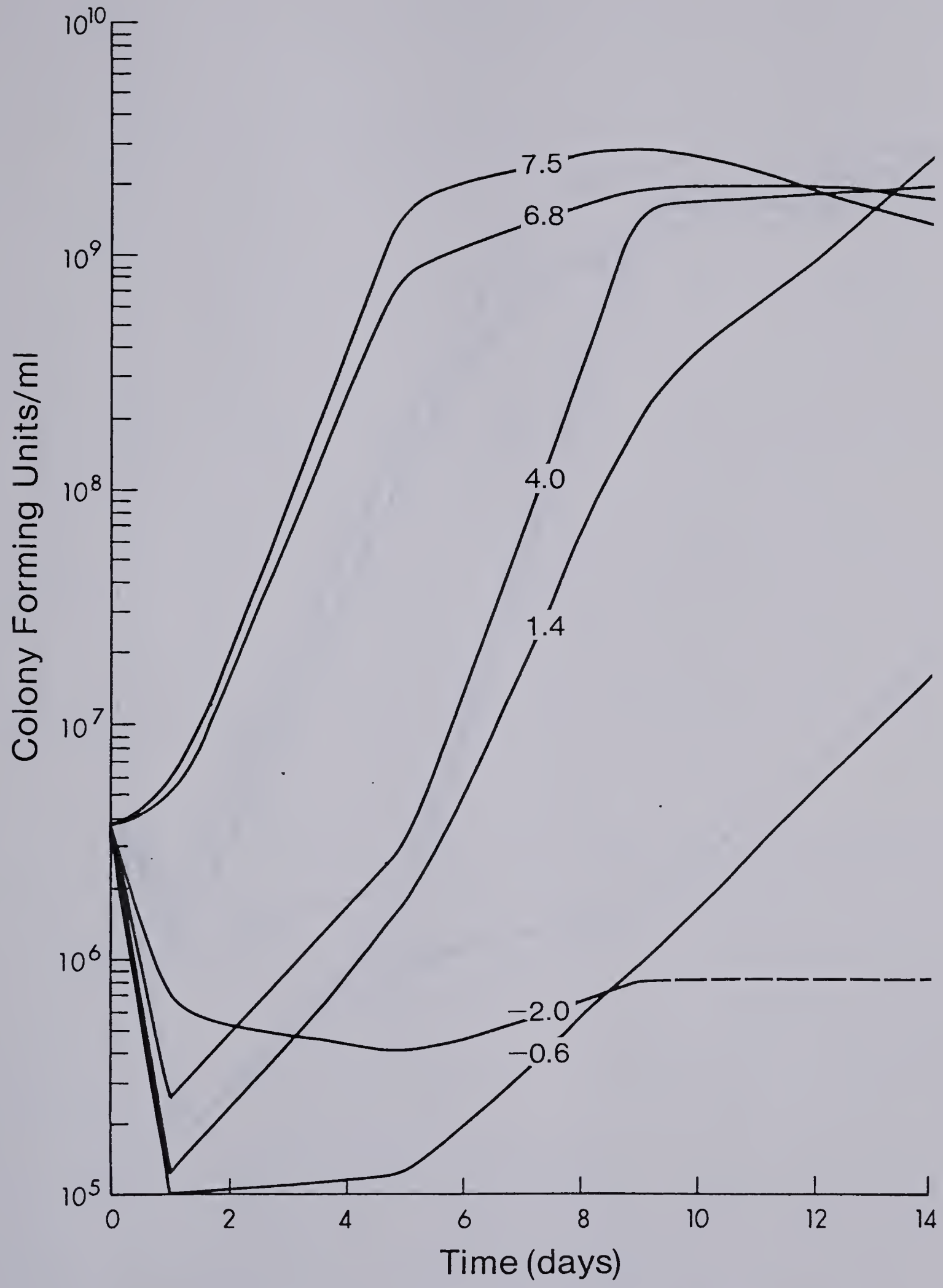


Fig. 12. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 7.0.

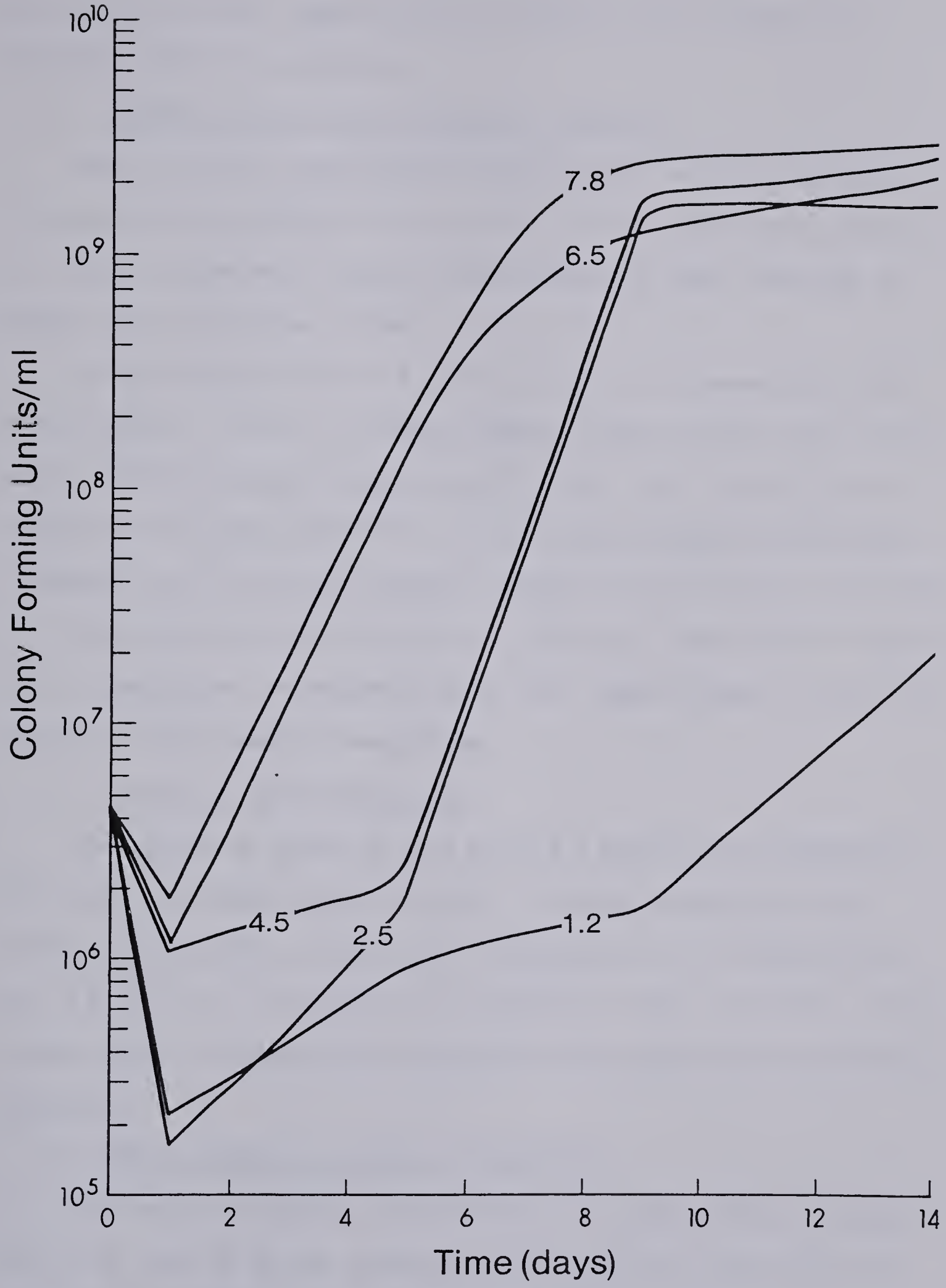


Fig. 13. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 9 in trypticase soy broth, pH 7.0.

most rapid at higher temperatures of incubation and in general was directly related to temperature.

B. Growth and Viability in TSB, pH 6.0 and 5.0

Due to similar growth patterns obtained for the three strains, *Y. enterocolitica* Serotype 0:3 was used in further experiments, because it is the serotype most frequently encountered in human infections in Canada (Toma and Lafleur, 1974).

Growth and viability in TSB, pH 6.0 and 5.0 is presented in Figs. 14, 15 and 16. The main difference between growth at pH 6.0 and 7.0 is seen in optimum growth temperature (25° v 33°C; Figs. 14 and 3), and minimum growth temperature (4° v 1.4°C). Maximum population densities at temperatures 32-40°C were greatly reduced at pH 6.0 (Figs. 14 and 15).

No growth occurred at pH 5.0 and there was a rapid loss of viability at all temperatures of incubation (Fig. 16). Rate of loss of viability increased with increasing temperature.

C. Minimum pH for Growth in TSB

The results on growth at pH 6.0 and 5.0 indicated the minimum pH for growth is between these two levels. In order to determine the minimum pH for growth, cultures were incubated at 37°C in TSB, pH 5.4, 5.5, 5.6 and 5.8. The results are presented in Figs. 17 and 18. It can be seen that the minimum pH for growth is pH 5.5 under these experimental conditions.

D. Effect of NaCl on Growth and Viability

The results on growth and viability of *Y. enterocolitica* Serotype 0:3 in TSB (pH 7.0) in the presence of added NaCl (5, 10 and 15%) are shown in Figs. 19-23. In the presence of 5% NaCl, growth occurred at

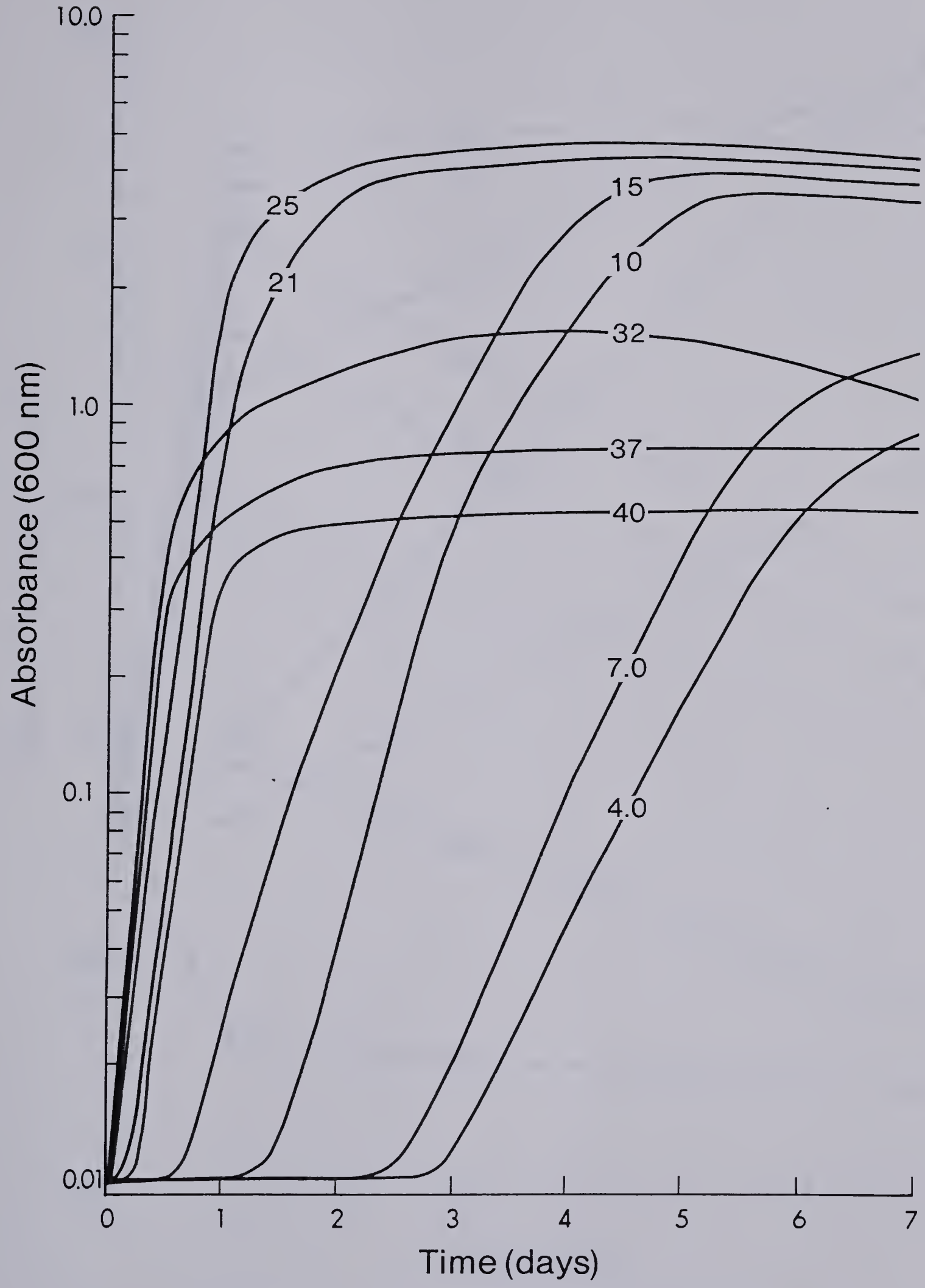


Fig. 14. Effect of temperature on growth of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 6.0.

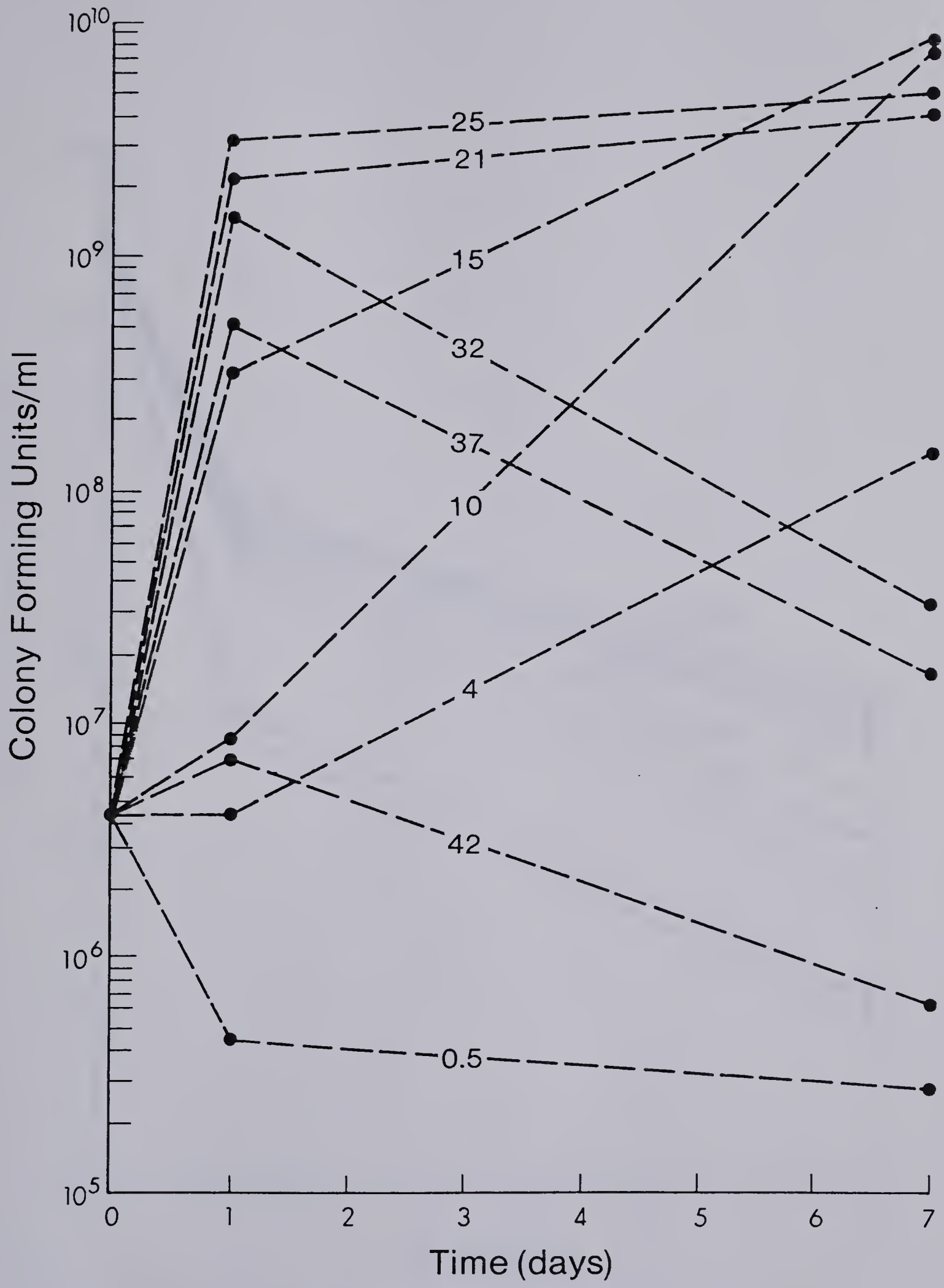


Fig. 15. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 6.0.

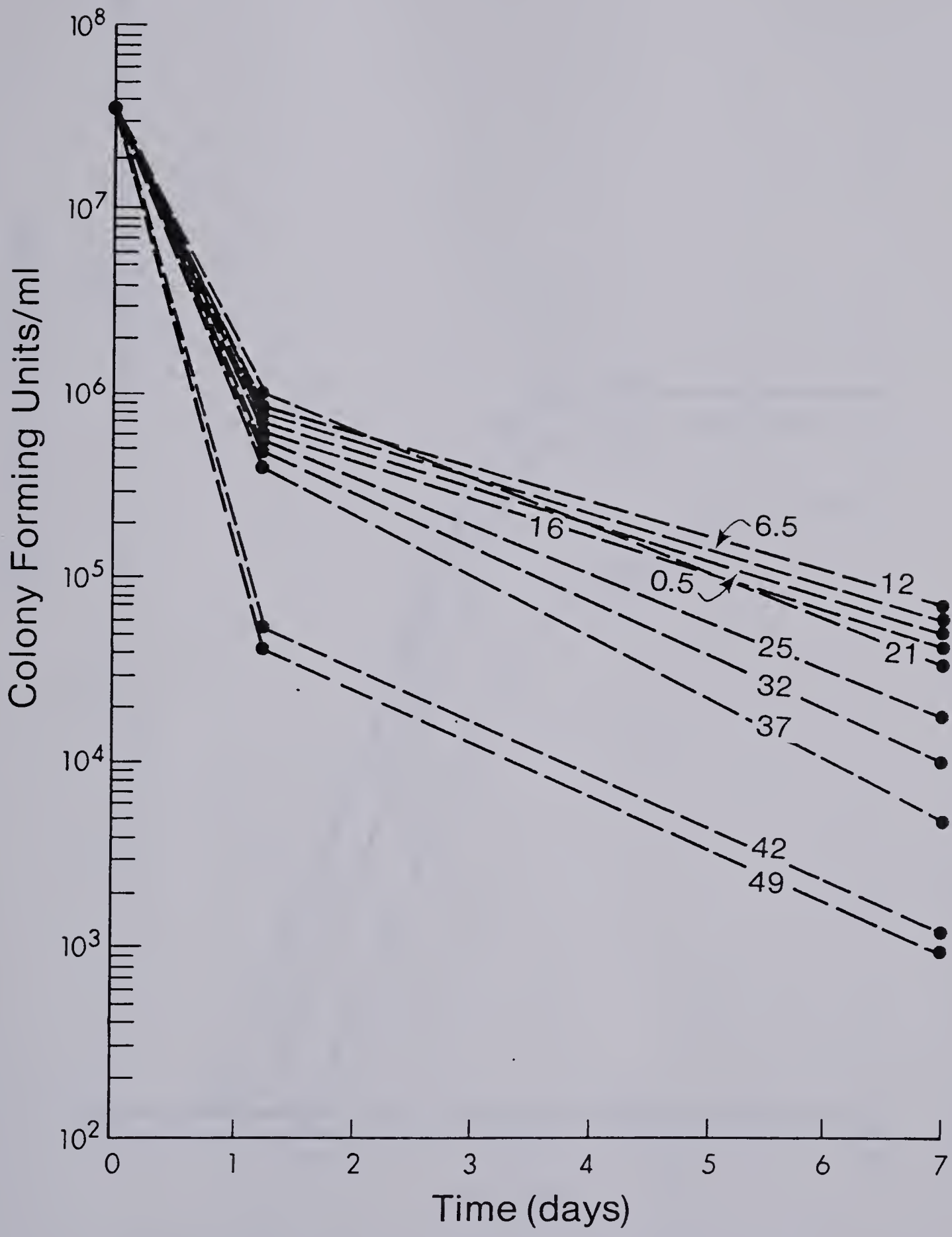


Fig. 16. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth, pH 5.0.

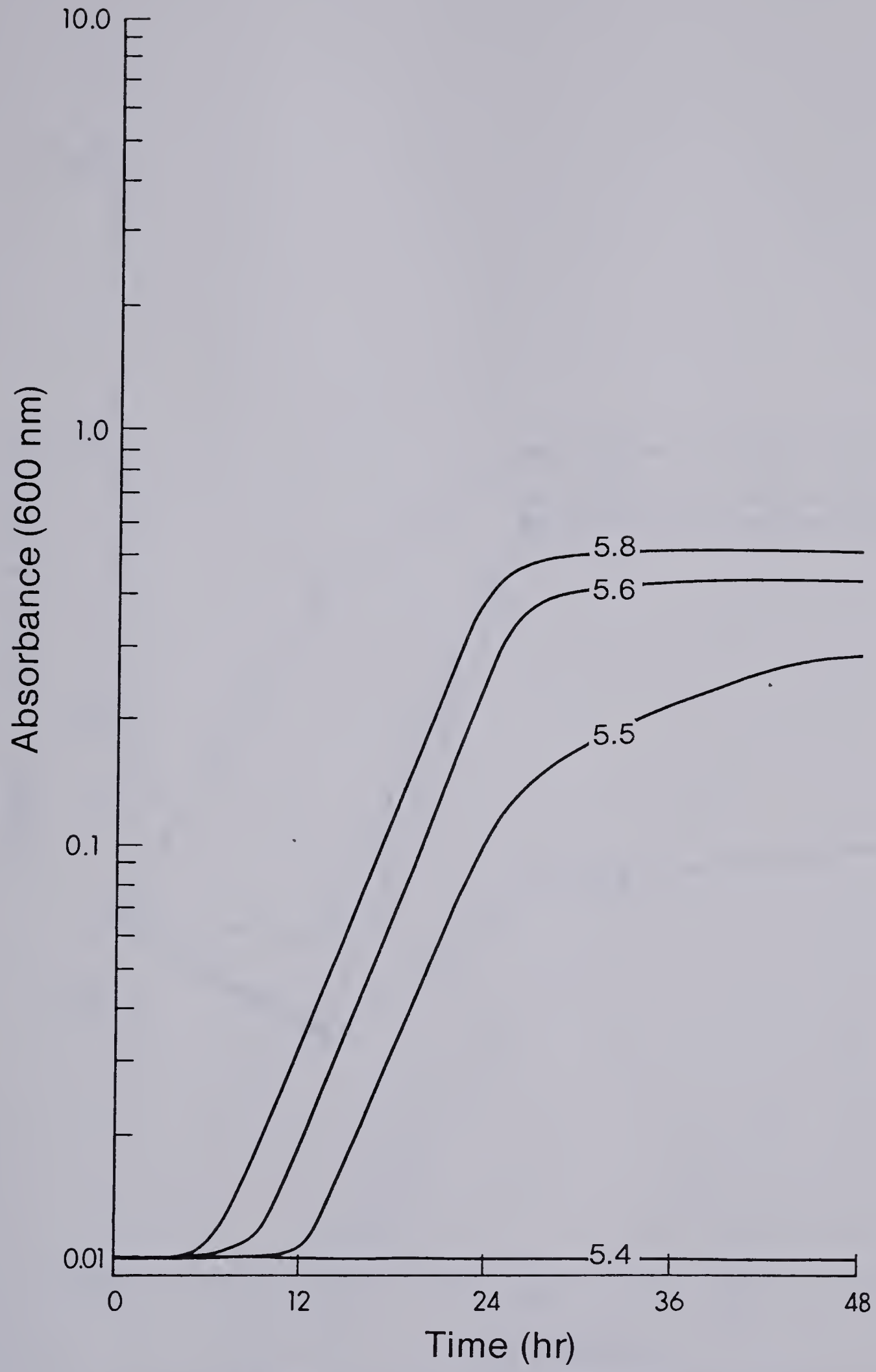


Fig. 17. Effect of pH (5.4-5.8) on growth of *Y. enterocolitica* Serotype 3 in trypticase soy broth at 37°C.

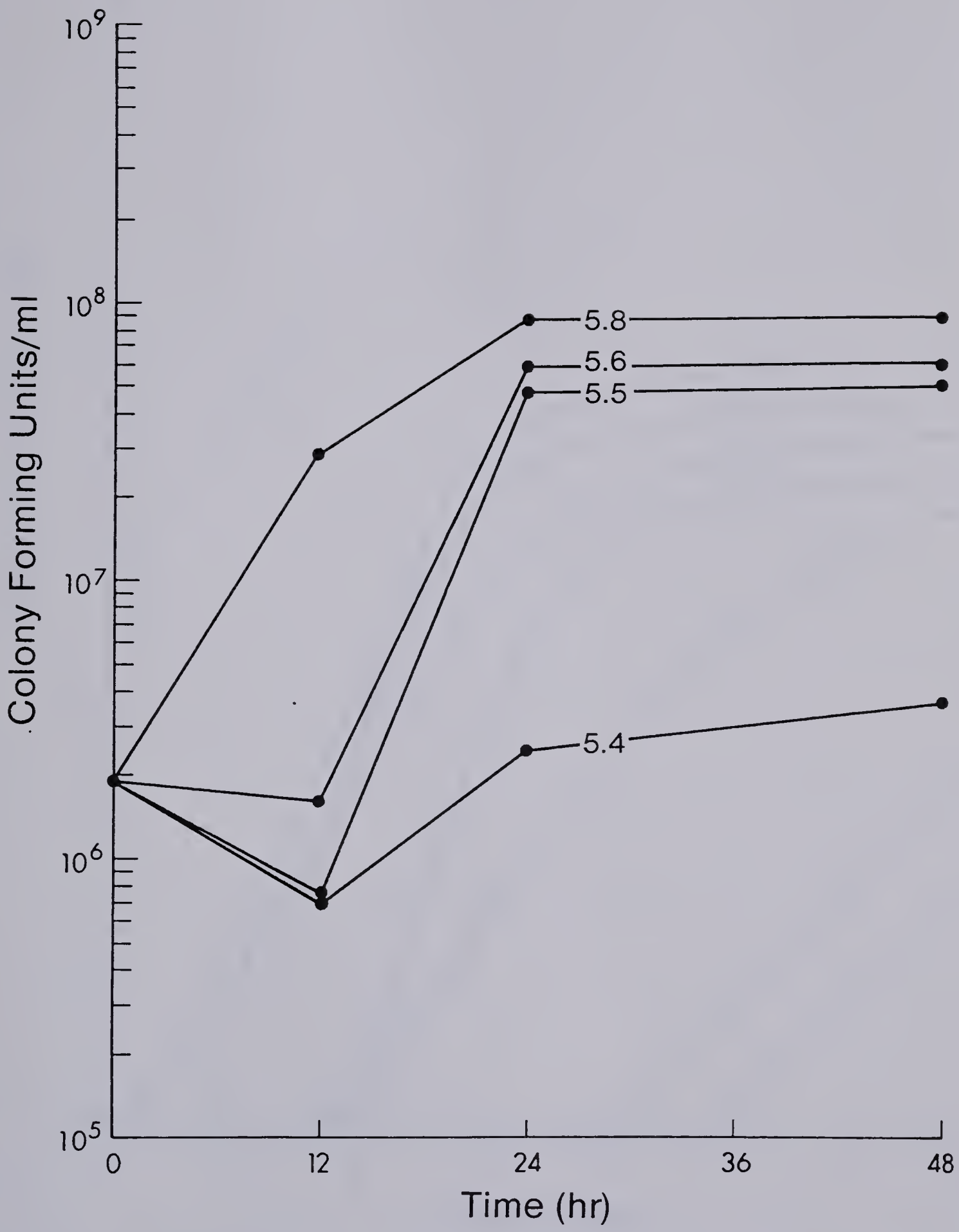


Fig. 18. Effect of pH (5.4-5.8) on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth at 37°C.

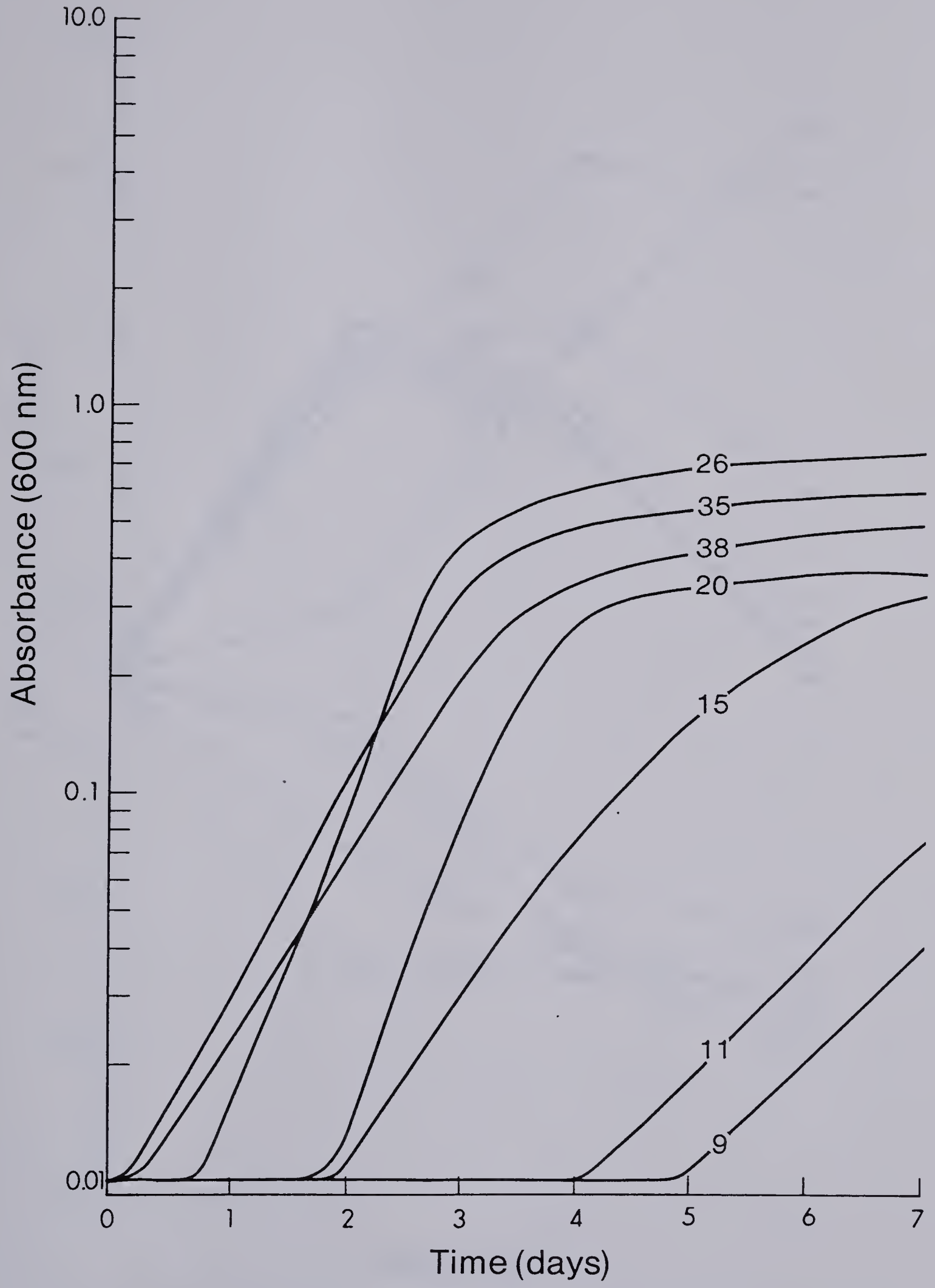


Fig. 19. Effect of temperature on growth of *Y. enterocolitica* Serotype 3 in trypticase soy broth, 5% sodium chloride, (pH 7.0).

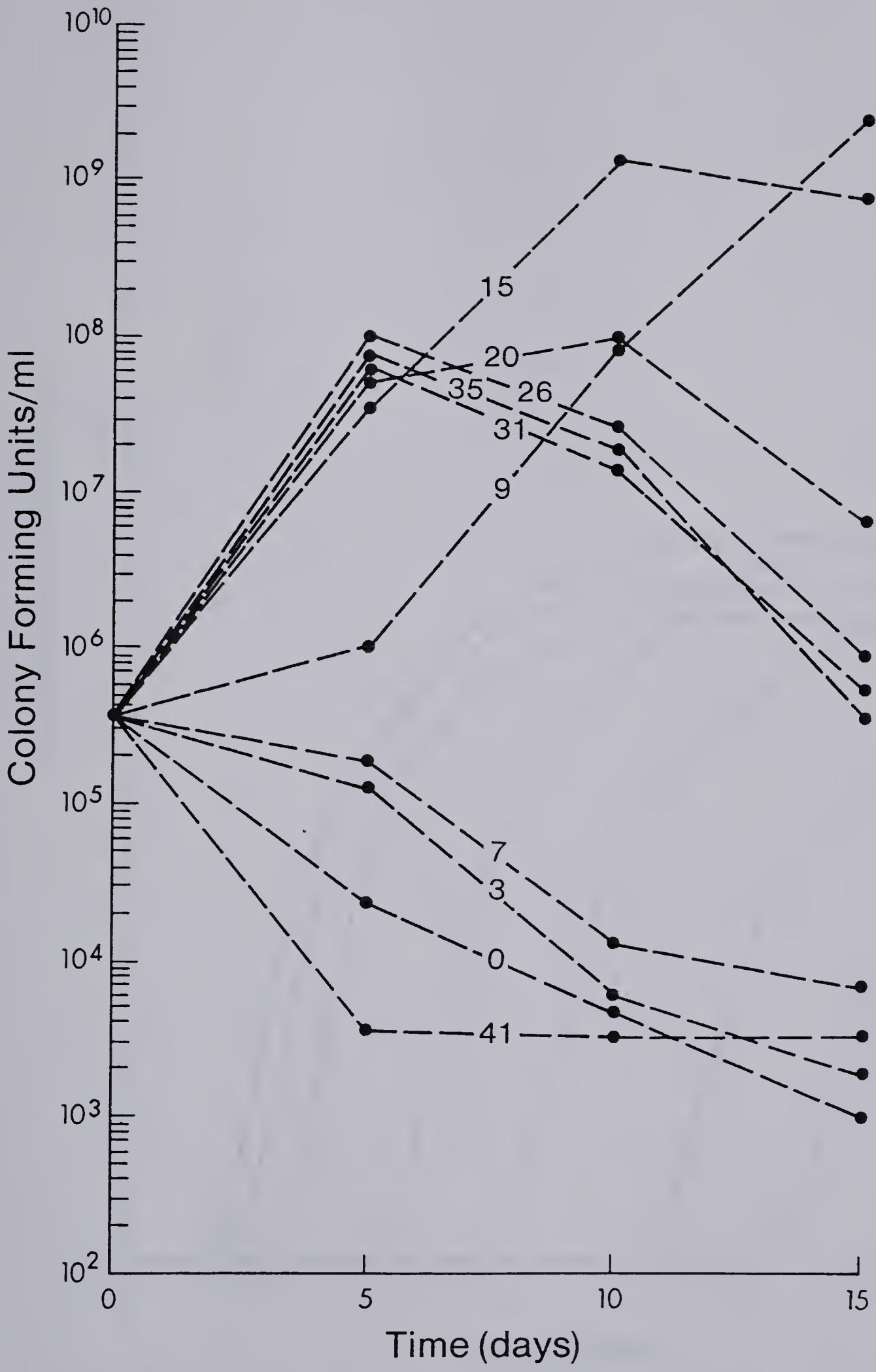


Fig. 20. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth, 5% sodium chloride, (pH 7.0).

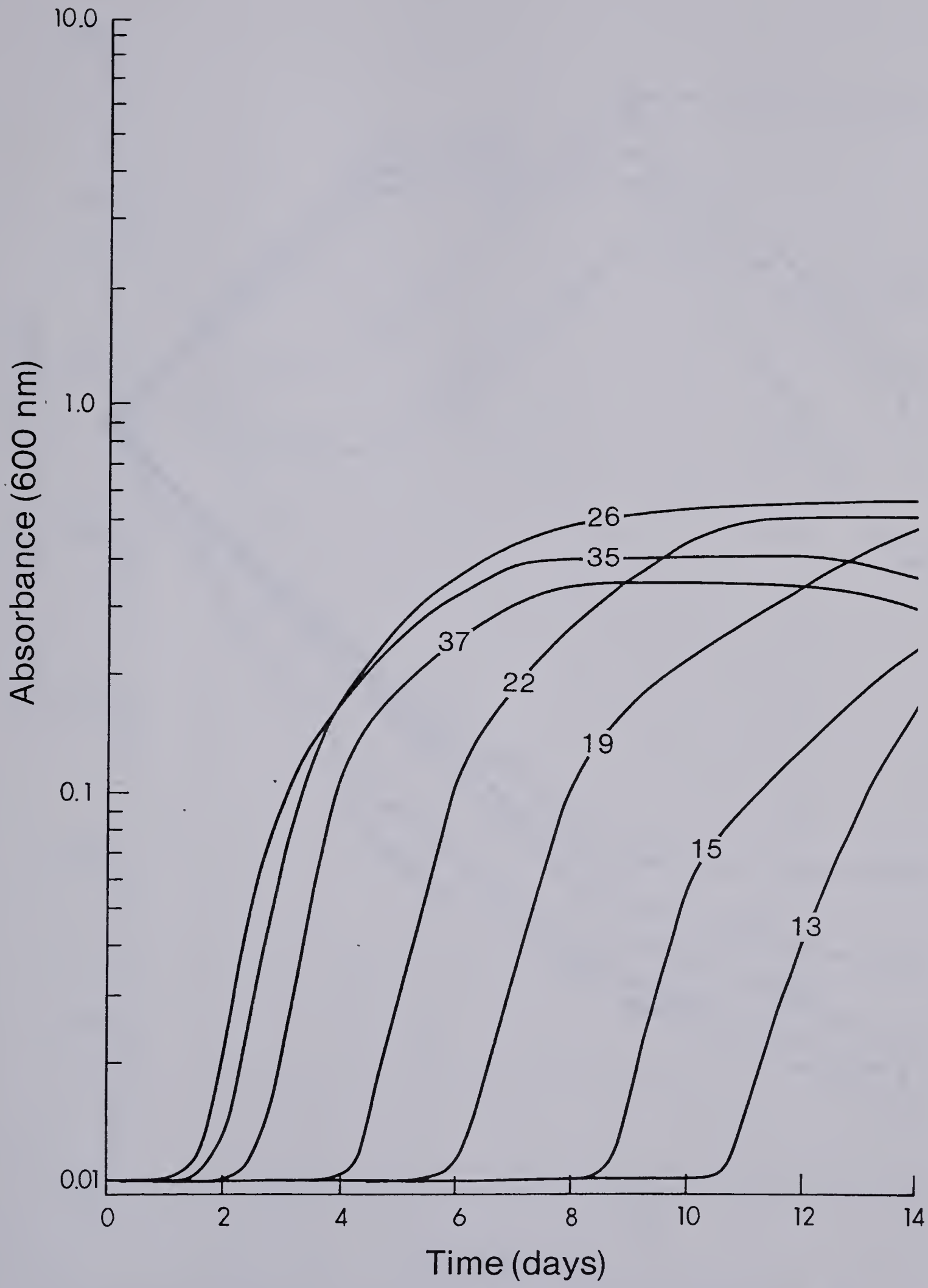


Fig. 21. Effect of temperature on growth of *Y. enterocolitica* Serotype 3 in trypticase soy broth, 10% sodium chloride, (pH 7.0).

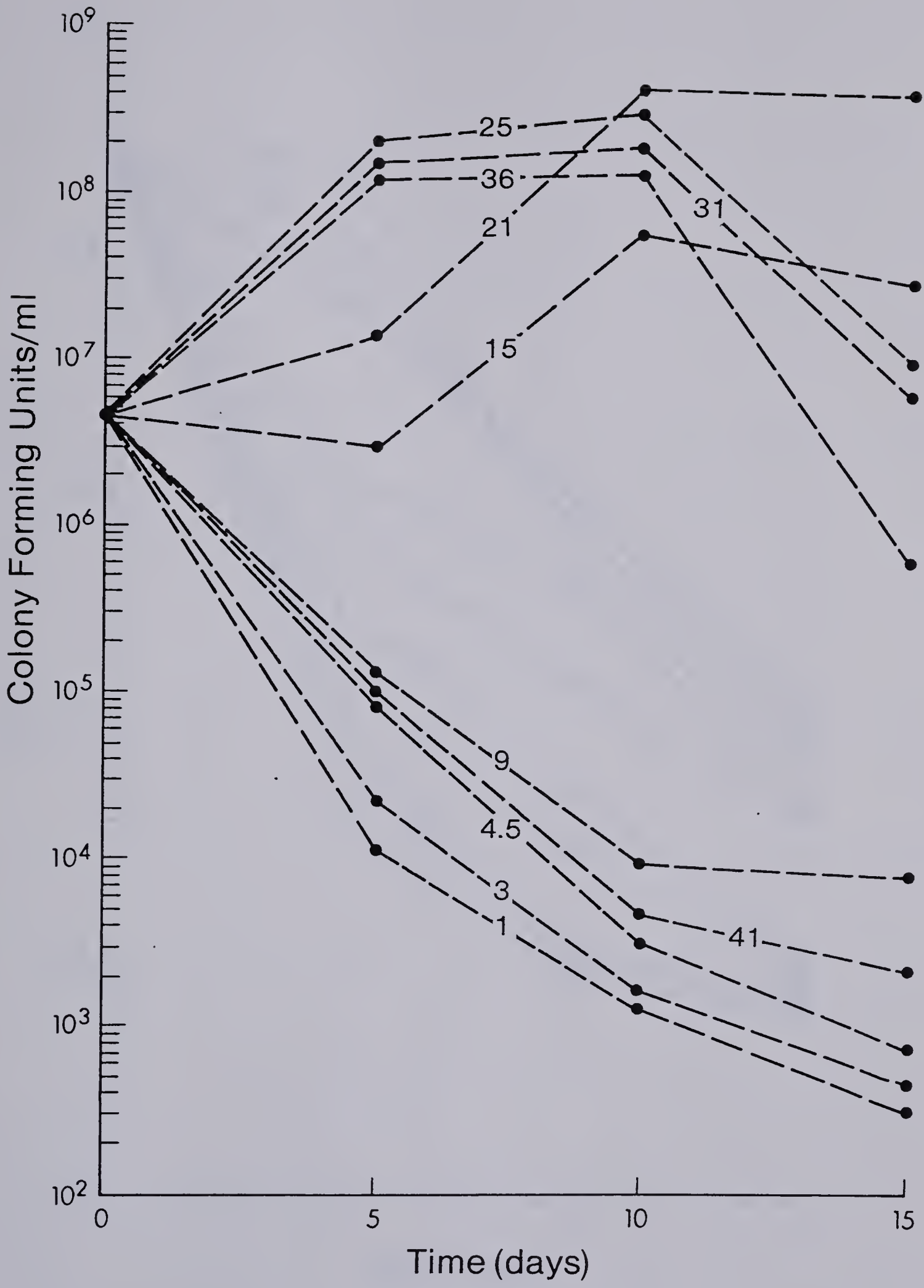


Fig. 22. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth, 10% sodium chloride, (pH 7.0).

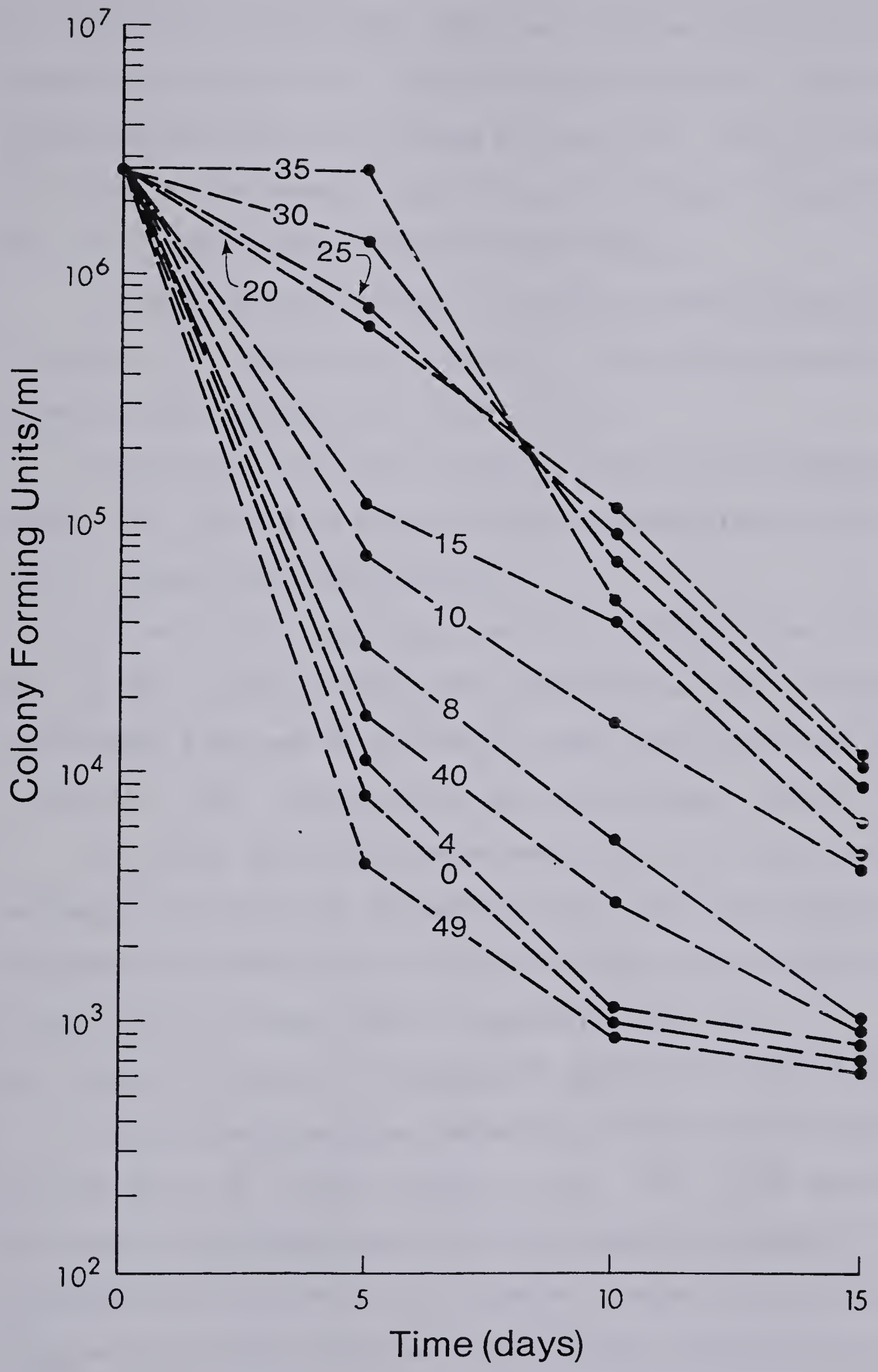


Fig. 23. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in trypticase soy broth, 15% sodium chloride, (pH 7.0).

all temperatures from 9-38°C. There was a gradual loss of viability at temperatures below 9°C (7, 3 and 0°C) and also at 41°C. Maximum population densities were achieved at 9 and 15°C. This is in contrast to results in the absence of NaCl (Fig. 9), although it should be noted the time to reach maximum levels is much longer.

The most noticeable effect of 10% NaCl is the extension of the lag phase of *Y. enterocolitica* (Fig. 21). The minimum temperature for growth in the presence of 10% NaCl is 13°C.

The presence of 15% NaCl inhibited growth at all temperatures from 0-40°C (Fig. 23) and all cultures showed a progressive loss of viability.

E. Growth and Viability in GSM

The results of growth and viability in GSM are shown in Figs. 24-28. At pH 7.0, the optimum growth temperature was 20-24°C and the minimum was 4.5°C and lag periods were much longer than those observed in TSB (Fig. 24). Maximum viable counts were found at 20-25°C (Fig. 25).

At pH 6.0, the optimum growth temperature was 15-20°C with a minimum of 6°C (Fig. 26) and maximum viable counts were found at 15-20°C. No growth was observed at pH 5.0 and there was a progressive loss of viability at all temperatures of incubation (Fig. 28).

2. *SELECTIVE ISOLATION AT REFRIGERATED TEMPERATURE USING ENTERIC MEDIA*

Yersinia enterocolitica has been reported capable of growing at 4°C (Feeley *et al.*, 1976; Highsmith *et al.*, 1977). This observation is the basis of cold temperature enrichment procedures presently in use for the selective isolation of this organism. However, such procedures require a very long incubation period, 28 days, before positive identification is possible. The results of the present investigation support

Fig. 24. Effect of temperature on growth of *Y. enterocolitica* Serotype 3 in glucose salts medium, pH 7.0.

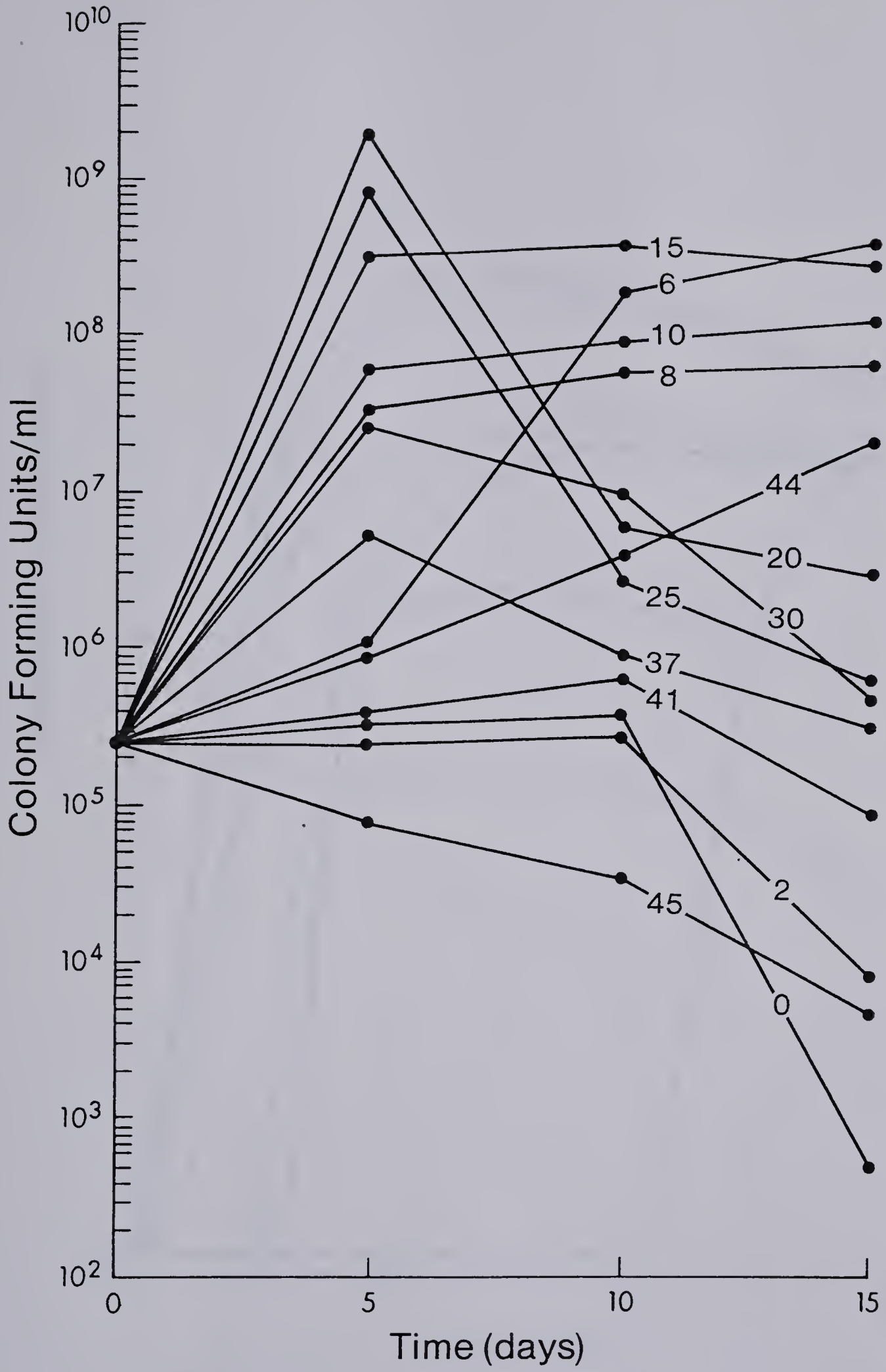


Fig. 25. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in glucose salts medium, pH 7.0.

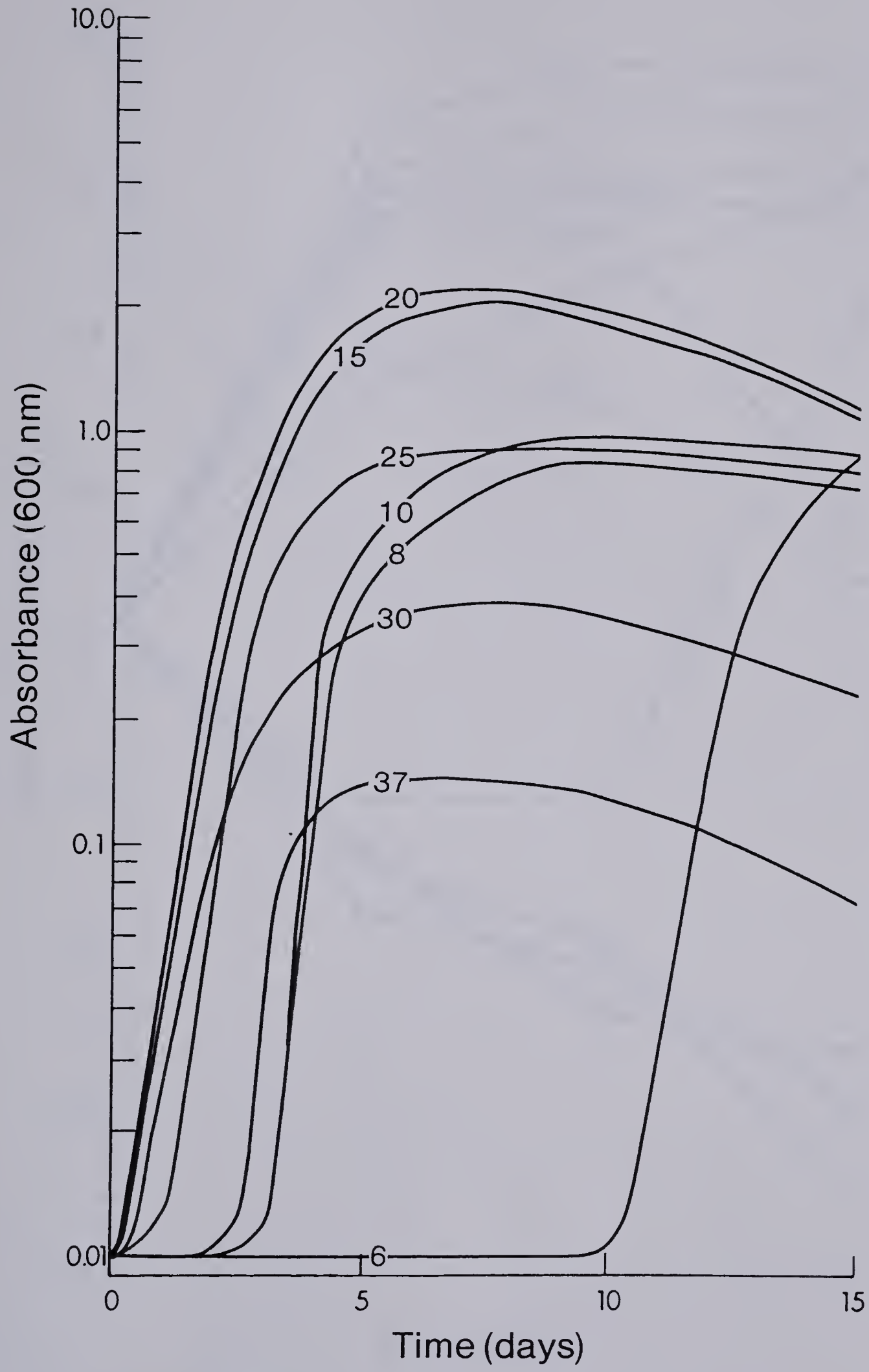


Fig. 26. Effect of temperature on growth of *Y. enterocolitica* Serotype 3 in glucose salts medium, pH 6.0.

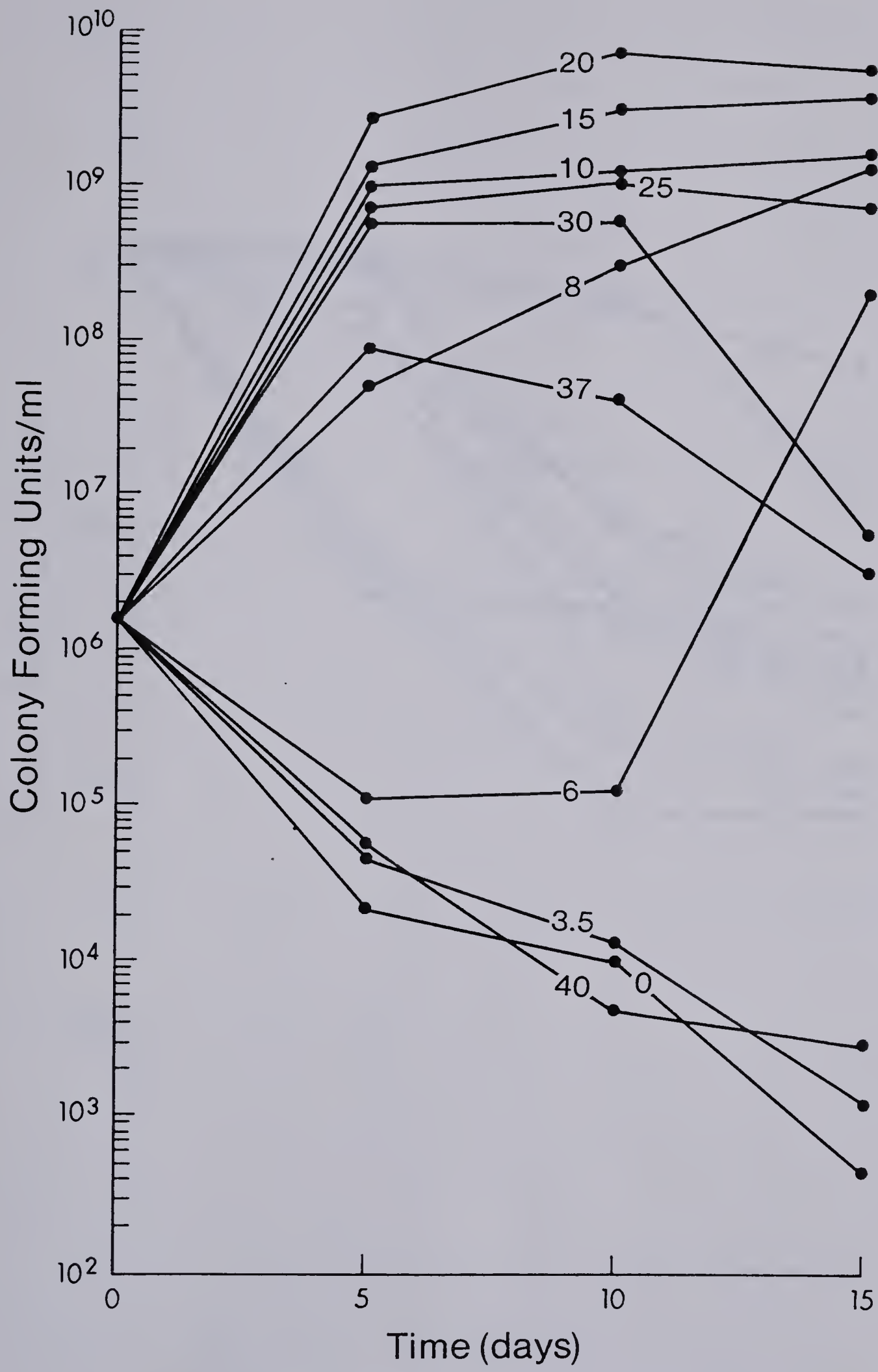


Fig. 27. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in glucose salts medium, pH 6.0.

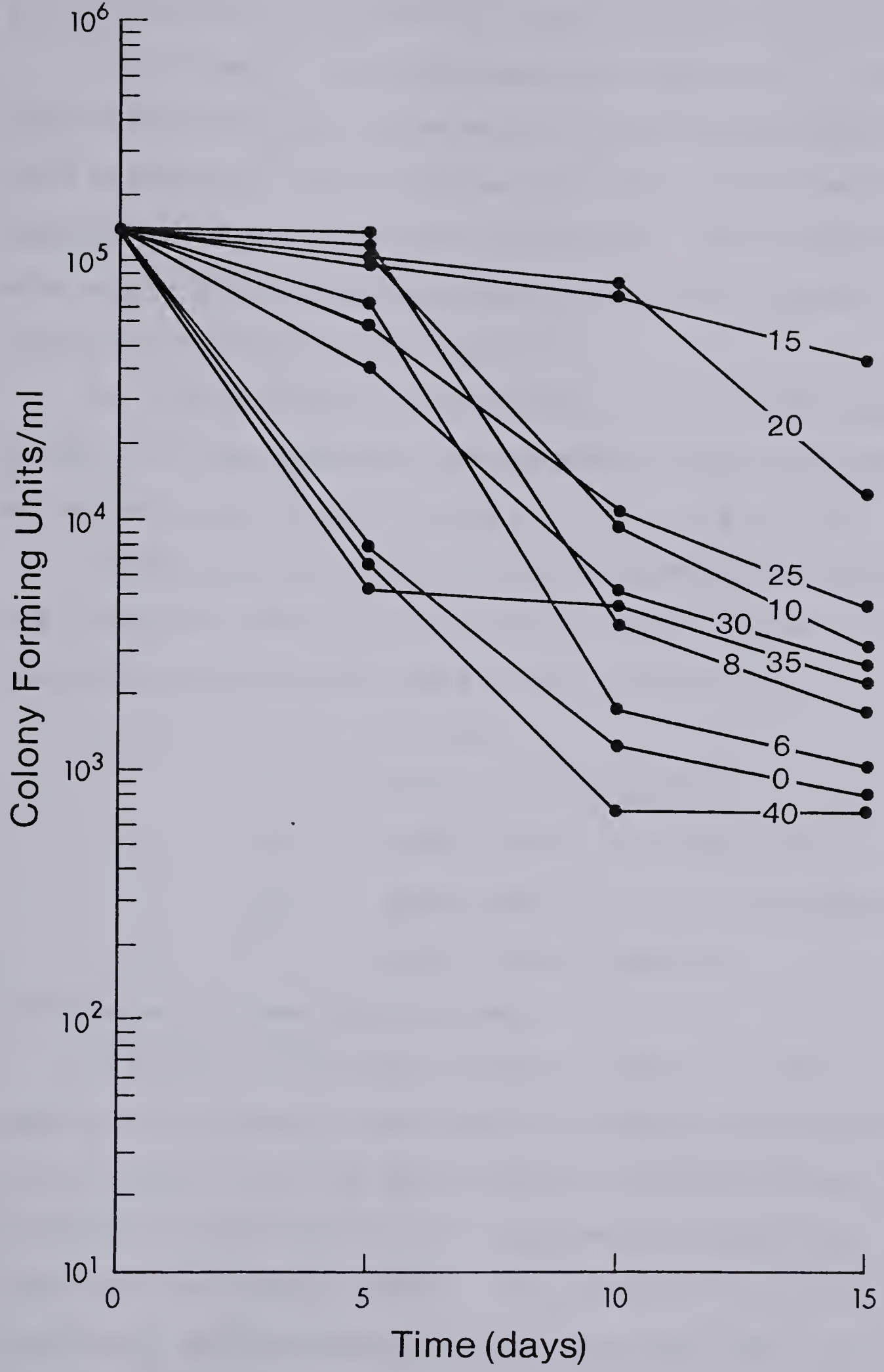


Fig. 28. Effect of temperature on growth and viability of *Y. enterocolitica* Serotype 3 in glucose salts medium, pH 5.0.

the earlier findings on growth of *Y. enterocolitica* at low temperatures.

In an attempt to hasten and improve the isolation of *Y. enterocolitica* it was felt that the capacity for growth at low temperature could be exploited. If the organism could grow on enteric media at low temperature, direct isolation could be possible. The inability of most other members of the *Enterobacteriaceae* to grow at refrigerated temperatures would support this hypothesis.

The first experiments were undertaken to establish the growth response of *Y. enterocolitica*, selected members of the *Enterobacteriaceae* and *Pseudomonas* sp. on enteric media at 3, 4, 5, 6, 8 and 10°C.

The agar plates were divided into four quadrants and streaked with each respective culture in order to obtain isolated colonies. Growth was crudely estimated and recorded in terms of (+) or (-).

(-) = no growth

(+) = growth over 1st quadrant

(++) = growth over 1st and 2nd quadrants

(+++) = growth over 1st, 2nd and 3rd quadrants

(++++) = growth over all quadrants

The plates were incubated up to 12 days.

The results of this study are shown in Table 2. Growth of the psychrophilic pseudomonad paralleled that of the *Y. enterocolitica* isolates, but this poses no major problems since both organisms could be presumptively differentiated on colony morphology and positively identified by their biochemical profiles. The cultures of the family *Enterobacteriaceae*, with the exception of *Ent. agglomerans* and *S. liquefaciens*, did not grow at temperatures below 8°C on enteric media. On the other hand,

TABLE 2

Growth of *Y. enterocolitica*, selected *Enterobacteriaceae* and *Pseudomonas* sp. at refrigerated temperatures on enteric media*

Cultures	3°C	4°C	5 & 6 °C	8 & 10°C
	9 days	7 days	5 days	3 days
*Y. enterocolitica***	++	++	+++	++
*Enterobacteriaceae****	-	-	-	+
Pseudomonas sp.	++	++	+++	+++

*Bismuth Sulfite (BS), Desoxycholate Citrate (DC), Eosin-Methylene Blue (EMB), Endo, MacConkey, Violet Red Bile (VRB), Violet Red Bile Glucose (VRBG).

**No growth observed on Brilliant Green and Salmonella-Shigella agars.

****Ent. agglomerans* and *S. liquefaciens* grew at all temperatures but colonies were detected 2 days later than those of *Y. enterocolitica*.

Y. enterocolitica did not grow on BG and SS agars. As a result of these observations, further experiments were limited to temperatures below 8°C and to the use of Endo, MacConkey, SS and VRBG agars.

A subsequent quantitative experiment at 6°C was undertaken in which test cultures were serially diluted and appropriate aliquots surface-plated on specific enteric agar. Results obtained are shown in Table 3. There was good recovery of both *Y. enterocolitica* isolates and the psychrophilic pseudomonad at 6°C for 5 days. 100% recovery rates were obtained on all media examined when the *Pseudomonas* sp. was used as the inoculum.

This is in contrast to results obtained with *Ent. agglomerans* and *S. liquefaciens*, both of which demonstrated a 0% recovery rate. Inoculum recovered for *Y. enterocolitica* varied from 85-100% on all media tested.

Owing to the wide array of commercially available enteric media and because of different recovery rates obtained in this endeavour, an evaluation of media was conducted. Results are shown on Table 4 which reflects the average recovery rates (%) for each medium tested. Although recovery rates on Endo, MacConkey, Violet Red Bile and Violet Red Bile Glucose media were all good, VRBG medium proved to be the most suitable all-purpose medium due to distinct morphological characteristics of colonies at test incubation temperatures. Colonies were distinctly large and bright pink in color due to the precipitation of bile salts present in the medium.

With *Y. enterocolitica*, growth on Brilliant Green and Salmonella-Shigella agar was inhibited (0% recovery rates) while growth of the

TABLE 3

Recovery of *Y. enterocolitica*, *Ent. agglomerans*, *S. liquefaciens* and *Pseudomonas* sp. when surface-plated on enteric media at 6°C for 5 days

Cultures	Inoculum Recovered (%)			
	Endo	MacConkey	SS	VRBG
Y. ent. ATCC 23715	100	85	0	100
0:3	86	87	0	85
0:9	93	96	0	95
0:5, 27	97	93	0	100
Ent. agglomerans	0	0	0	0
S. liquefaciens	0	0	0	0
Pseudomonas sp.	100	100	100	100

TABLE 4

Medium evaluation indicating recovery rates (%) of *Y. enterocolitica* serotypes* and *Pseudomonas* sp.** grown at 6°C for 5 days

Medium	Inoculum Recovered (%)				
	Y. ent. ATCC 23715	*Y.e.* 0:3	*Y.e.* 0:9	*Y.e.* 0.5,27	*Pseudomonas* sp.
Bismuth Sulfite	55	77	69	55	NT***
Brilliant Green	0	0	0	0	NT
Deoxycholate Citrate	43	62	67	69	NT
Eosin-Methylene Blue	66	55	78	84	NT
Endo	100	86	93	97	100
MacConkey	85	87	96	93	100
Salmonella-Shigella	0	0	0	0	100
Violet Red Bile	83	85	93	95	NT
Violet Red Bile Glucose	100	99	99	100	100

*Initial inoculum 2 x 10^3 c.f.u./ml.

**Initial inoculum 5 x 10^2 c.f.u./ml.

***Not Tested.

psychrophilic pseudomonad was not inhibited on SS agar (100% recovery rate).

SS agar is one of the media recommended for the isolation of *Y. enterocolitica* at 25°C for 48 hrs by Feeley *et al.* (1976) in the "Compendium of Methods for the Microbiological Examination of Foods" prepared by the American Public Health Association (APHA) and edited by M. Speck. However, at refrigerated temperatures *Y. enterocolitica* appears to be sensitive to some agent or agents in the SS agar and as a result of this finding, an experiment was designed to define the growth-limits on SS agar of this organism. A temperature range of 5 to 45°C was established and aliquots of appropriate dilutions of the test cultures were surface-plated onto SS and VRBG agar. Table 5 indicates results obtained. There was a 100% recovery rate at temperatures 16-40°C but a 0% recovery rate at 15°C on SS agar. VRBG agar was included for comparative purposes and a 100% recovery rate was demonstrated on this medium at 5-40°C. From this experiment, it is apparent that 15°C is the critical temperature below which *Y. enterocolitica* indicates sensitivity to a selective agent or agents incorporated in the SS formula.

3. *DEVELOPMENT OF Y. ENTEROCOLITICA INOCULATED ONTO RAW PORK SAMPLES*

The ability of *Y. enterocolitica* to grow on raw pork samples at 0, 5 and 10°C was investigated. In addition, selective isolation on Violet Red Bile Glucose (VRBG) and Bismuth Sulfite (BS) agars was studied.

The inoculum was grown in sterile TSB at 37°C for 18 hrs and sub-cultured twice. Pork samples weighing approximately 10g were inoculated

TABLE 5

Growth limits of *Y. enterocolitica* on SS agar and VRBG agar

Temperature (°C)	Inoculum recovered (%)	
	SS	VRBG
45	0	0
40	100	100
37	100	100
30	100	100
25	100	100
20	100	100
18	100	100
16	100	100
15	0	100
10	0	100
5	0	100

with *Y. enterocolitica* by spreading 0.1 ml inoculum over the upper surface. This yielded approximately 3.6 x 10^3 c.f.u./gram of meat. Inoculated samples were placed in sterile polythene bags and incubated at test temperatures for a period of 12 days. At each sampling interval, duplicate bags were removed and each sample was "stomached" for 3 mins with 90 mls sterile peptone water (0.1%). Appropriate decimal dilutions of the homogenate were made in sterile peptone water and aliquots surface-plated in triplicate on TSA plates incubated at 37°C for 2 days and on VRBG and BS agar plates incubated at 5°C for 10 days.

Results of *Y. enterocolitica*, as determined by confirmed colony counts on TSA, on raw pork stored at 0, 5 and 10°C are presented in Fig. 29. Growth occurred at all test temperatures under study. However, growth of the organism was most extensive after 8 days incubation with little to moderate increases in counts after a 12-day incubation period.

In order to determine the potential value of low temperature isolation of *Y. enterocolitica*, inoculated samples of pork stored at 0, 5 and 10°C were plated on VRBG and BS agars. The plates were incubated at 5°C for 10 days. The identity of representative colonies growing on the plates was confirmed by biochemical tests. The results of this experiment are shown in Fig. 30. It can be seen that recovery on VRBG was consistently higher than recovery on BS at all temperatures. The recovery compares fairly closely to numbers of *Y. enterocolitica* as determined in a previous experiment (Fig. 29). The recovery of *Y. enterocolitica* on VRBG agar varied between 50-80% and BS agar between 50-65% when compared to counts obtained on TSA. The higher recovery rates were obtained at 10°C.

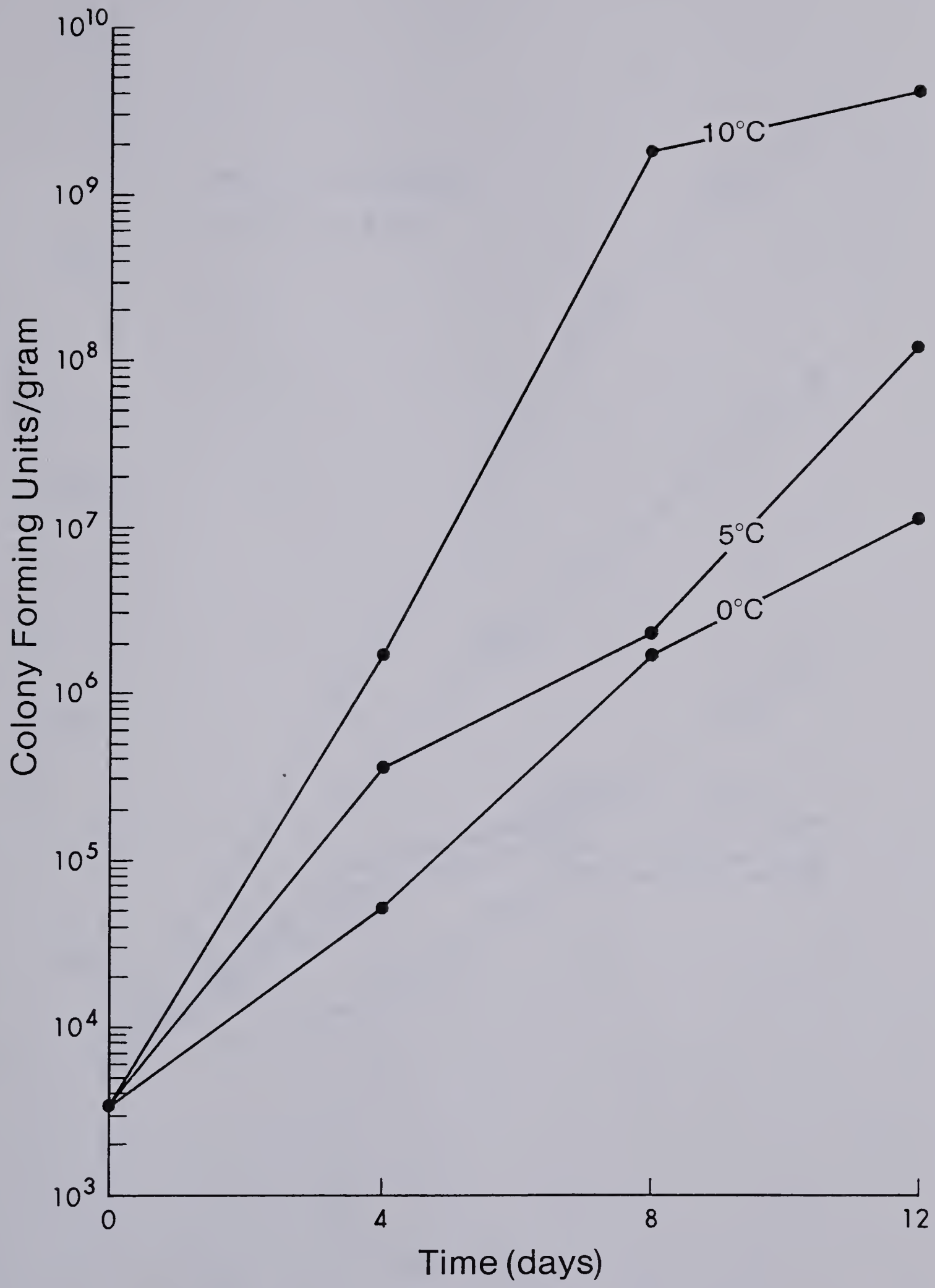


Fig. 29. Growth of *Y. enterocolitica* Serotype 3 on raw pork at 0, 5 and 10°C.

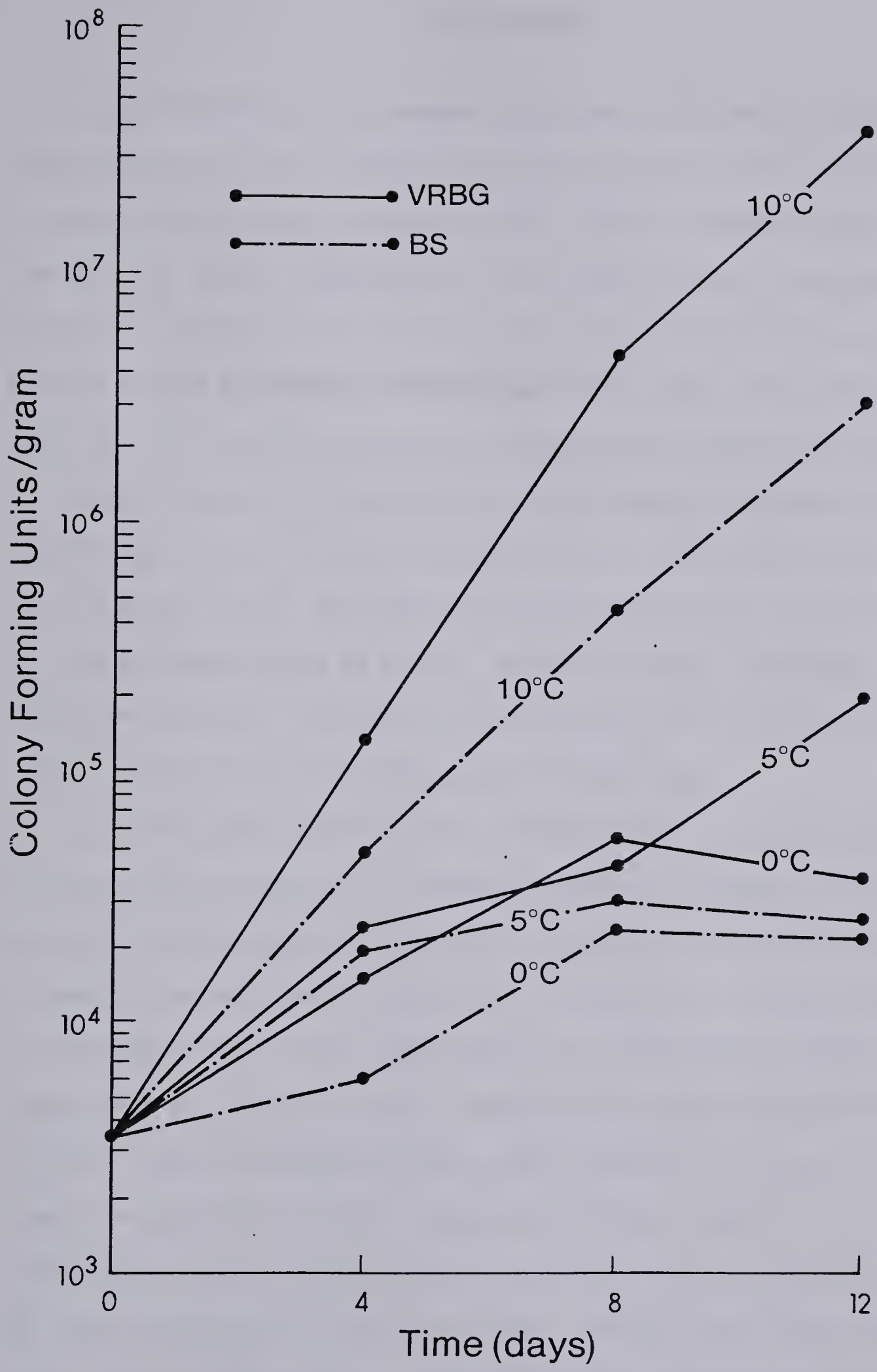


Fig. 30. Selective isolation of *Y. enterocolitica* Serotype 3 from raw pork on violet red bile glucose and bismuth sulfite agars.

DISCUSSION

The objective of the present study was to determine temperature relationships of three strains of *Yersinia enterocolitica* when grown in a complex medium (TSB), in adjusted TSB, and in a minimal medium, GSM. The cardinal growth temperatures of the three strains, as determined by absorbance, compared very closely when grown in TSB (pH 7.0). Lowest minimum growth temperature recorded was 0.5°C after 7 days incubation (Fig. 2). The results obtained on minimum growth temperature for *Y. enterocolitica* are in agreement with minimum growth-temperature limits reported by most other workers (Feeley *et al.*, 1976; Highsmith *et al.*, 1977; and Lee, 1977), who reported on the ability of *Y. enterocolitica* to grow at temperatures of 0-4°C. Results obtained, therefore, confirm and extend previous findings and observations on the ability of *Y. enterocolitica* to grow at refrigerated temperatures.

The data from Figure 7 would indicate that *Y. enterocolitica* shows the temperature response of a typical mesophile; however, this organism may be considered atypical in that it clearly demonstrates the capacity to grow at temperatures as low as 0°C. Based on our present knowledge of reported minimum growth temperatures for food-borne pathogens it would appear that *Y. enterocolitica* is possibly the human food-borne pathogen with the lowest reported minimum growth temperature. Known minimum growth temperatures for other pathogens include a publication by Jackson (1974) reporting a minimum growth temperature ranging from 9.5-10.5°C for eight strains of *V. parahaemolyticus*. Matches and Liston (1968) also reported minimum growth temperatures for *Salmonella heidelberg*,

S. typhimurium and *S. derby* as 5.3, 6.2 and 6.9°C respectively when grown in broth culture after 19 days incubation. Ingram and Mackay (1976) reported the observation of Schmidt *et al.* (1961) that *Clostridium botulinum* Type E grew in beef stew at a temperature of 3.3°C.

Knowledge of minimum growth temperatures for most food-borne pathogens has some practical implications since such findings may determine regulations and recommendations designed to keep foods safe and free from bacterial growth. For example, recommendations that smoked (Olson, 1968) or irradiated (Anon., 1966) fish should be stored below 3°C are based on the observation of Schmidt *et al.* (1961) that growth of *Cl. botulinum* Type E occurred down to 3.3°C.

It is imperative at this point to emphasize the fact that different environmental factors are likely to affect the true minimum temperature for growth, and that "the true minimum temperature for growth of an organism could be determined only if all other factors affecting growth were optimal" (Michener and Elliott, 1964). Such a statement indicates that it may not always be necessary to take the lowest growth temperature ever recorded for an organism and use this as an absolute reference point, since all other factors affecting growth may not be optimal.

Nutrient status, water activity, and acidity are some external or environmental factors affecting the minimum growth temperature of organisms. This subject is well reviewed by Ingram and Mackey (1976) therefore further discussion on such factors will, for most part, be restricted to data obtained in this study on *Y. enterocolitica* Serotype 0:3.

a. *Nutrient Status*

The minimum temperature for growth of an organism is believed to

be lower if suitable nutrients are present in the substrate (Ingram and Mackey, 1976), hence, minimum growth temperatures depend on the nutritive status of the menstruum. For instance, minimum growth temperature for *Y. enterocolitica* was recorded at 1.4°C over a 7-day incubation period in the complex medium, TSB (Fig. 4), while the growth limit of the same organism in the minimal medium, GSM, was higher (4.5°C) over a 10-day incubation period (Fig. 23). Likewise, Schmidt *et al.* (1961) recorded a minimum growth temperature of 3.3°C in beef stew for *Cl. botulinum* Type E, while the growth limit was higher in peptone water (8°C).

b. *Water Activity* (a_w)

Figures 19 and 21 clearly demonstrate the influence of reduced water activity on minimum growth temperature of *Y. enterocolitica*. The presence of added solute (NaCl) reduces or lowers the water activity resulting in restricted growth temperatures of the organism. Minimum growth temperature of *Y. enterocolitica* in TSB was raised from 1.4°C to 9°C (TSB + 5% NaCl) to 13°C (TSB + 10% NaCl) with no recorded growth at 0-40°C in TSB + 15% NaCl. These findings are in agreement with work performed by Segner *et al.* (1966) on *Cl. botulinum* Type E who found that 4.0% NaCl, which had little effect on growth at 30°C, led to extremely long lag periods at 8°C; and with 4.5% NaCl, growth did not take place at 8°C, but did so at 16°C. Ohye *et al.* (1966) likewise recorded growth up to 5.8% NaCl at 30°C, 5.1% at 20°C and 4.3% at 15°C. More detailed confirmatory observations with *Cl. botulinum* Type E were made by Emodi and Lechowich (1969) using various solutes besides NaCl to control a_w. There were differences in solutes in detail, but the same general relation was held throughout. It was also observed with

three salmonellae by Matches and Liston (1972), with *S. heidelberg* for example, growth was possible up to only 2% NaCl at 8°C, to 6% at 12°C, and to greater than 8% at 22-41°C. Similar observations were made by these researchers at slightly lower salt concentrations with *S. derby* and *S. typhimurium.*

c. *Acidity*

It is generally considered that suboptimal pH leads to a higher minimum growth temperature (Ingram and Mackey, 1976). This observation is reflected in results obtained when *Y. enterocolitica* was grown in TSB and GSM at varying pH levels. The minimum growth temperature recorded in TSB pH 7 and 6 was 1.4° and 4.0°C, respectively (Fig. 4 and 14). At pH 5.0, no growth of *Y. enterocolitica* was detected at temperatures of 0-45°C. In GSM, the same trend was observed - the lower the pH, the higher the minimum temperature for growth. Matches and Liston (1972) also made similar observations with three *Salmonella* serotypes. They observed a narrowing of the pH range near the minimum for growth.

It must be recognized that the results obtained on low temperature growth were determined under a limited set of laboratory conditions. In addition, it must be emphasized that erroneous estimates of minimum growth temperatures of organisms may result through observation for too short a period of time. Different results may have been obtained with longer incubation times. Estimation of true minimum growth temperatures is clearly a complex subject that merits more detailed and explicit attention.

Because it was observed in previous experiments (Figs. 2, 4 and 6) that *Y. enterocolitica* grew at refrigerated temperatures, a primary

objective of this investigation was to determine and establish growth response of *Y. enterocolitica*, selected members of *Enterobacteriaceae* and a psychrophilic pseudomonad at low temperature using commercially available enteric media. It was thought that if *Y. enterocolitica* could grow on enteric media at refrigerated temperatures, with little or no competition from other members of *Enterobacteriaceae*, then it might be possible to isolate *Y. enterocolitica* directly in this way.

Presently, a wide selection of enteric media is commercially available, but when utilized for the detection of *Enterobacteriaceae*, incubation temperatures adopted are usually 25°, 37° and 42°C for no longer than 2 days. However, in this study, low temperature incubation of plates was used over longer periods, and no apparent loss of sensitivity of the media was detected since high recovery rates were obtained for most of the media studied (Table 3).

Results indicate that selective isolation of cultured *Y. enterocolitica* is possible using enteric media incubated at 6°C for 5 days. Although growth of the psychrophilic pseudomonad paralleled that of *Y. enterocolitica* at all temperatures studied, it should be noted that it is possible to presumptively differentiate these two organisms on colony morphology and confirm by use of biochemical tests. The oxidase test is a key test that could be used to differentiate both organisms since *Y. enterocolitica* lacks this enzyme. Based on previous and present laboratory experience, the author would like to add that it was possible, with time, to visually differentiate *Y. enterocolitica* colonies from *Pseudomonas* sp. and from other enterobacterial colonies that grew on VRBG agar medium incubated at low temperatures. Colonies could

be presumptively differentiated on characteristics such as size, color, consistency and on diameter of precipitation ring surrounding each colony.

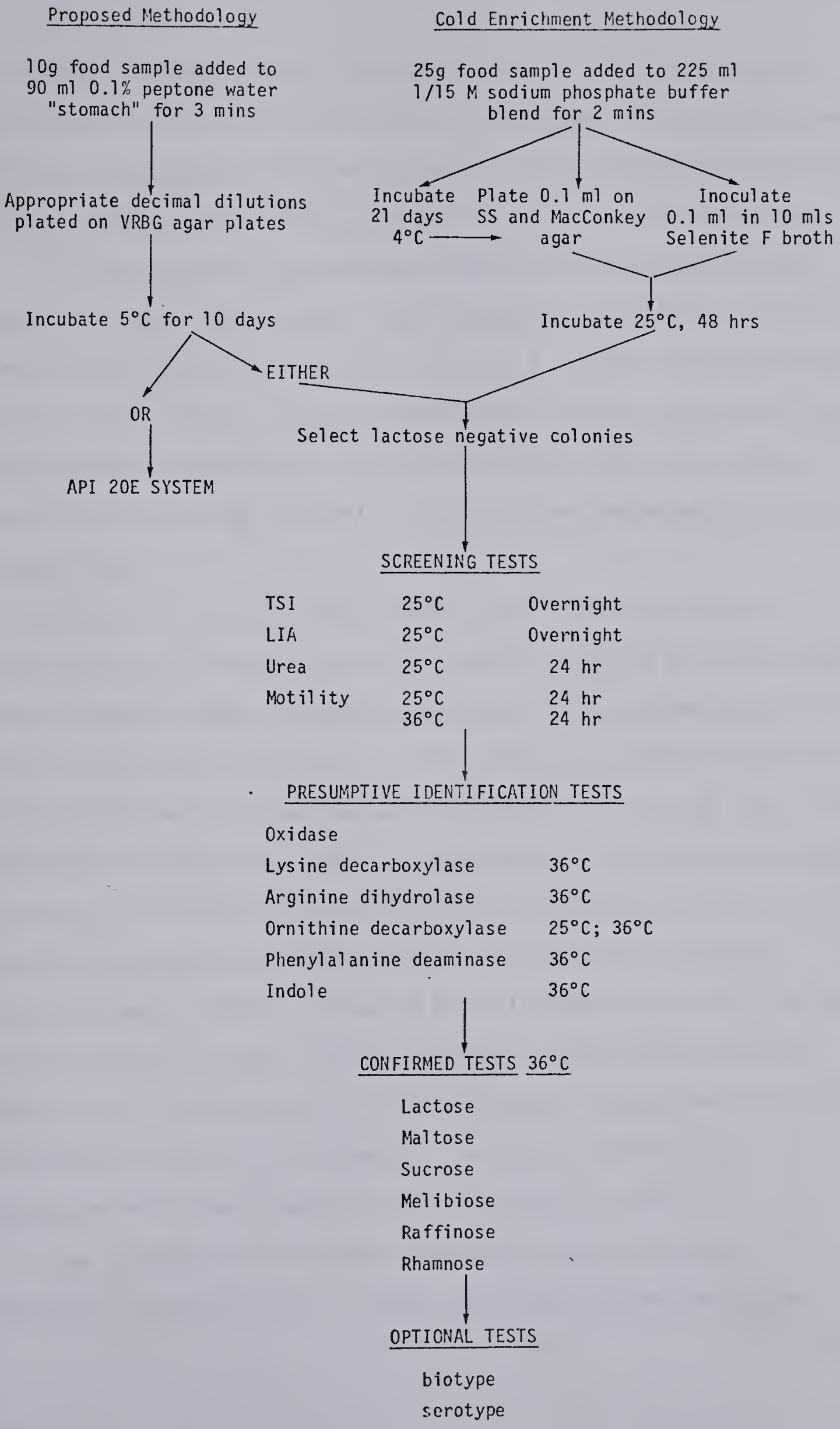
The results of this study also suggest that incubation of enteric plates at 6°C for 5 days is not absolutely selective for *Y. enterocolitica* since growth of other organisms occurred at this temperature (Tables 1 and 2). Nonetheless, the proposed low temperature isolation procedure provides a methodology that could be used together with cold enrichment procedures currently in use for the isolation and identification of *Y. enterocolitica.*

In using the proposed methodology described in Fig. 31, it should be noted that much time and materials could be saved since the need for pre-enrichment broth and additional use of media at specific intervals is eliminated.

Indications are that VRBG agar has potential as the enteric medium to be utilized for the low temperature isolation of *Y. enterocolitica.* Like many other enteric media utilized today in the clinical and food laboratories, it is possible that refinement of this medium - e.g. alteration of the pH; addition of indicators or other ingredients, might yield more positive identification of *Y. enterocolitica* and, at the same time, restrict growth of other unwanted *Enterobacteriaceae.* If this is accomplished, then it would be necessary to investigate its feasibility on a wide variety of foods - particularly meats from different environments in order to determine the effects on different interfering microbes.

In a preliminary experiment it was observed that counts on VRBG

FIGURE 31


Proposed Methodology
10g food sample added to 90 ml 0.1% peptone water "stomach" for 3 mins
Appropriate decimal dilutions plated on VRBG agar plates
Incubate 5°C for 10 days
OR
API 20E SYSTEM
Cold Enrichment Methodology
25g food sample added to 225 ml 1/15 M sodium phosphate buffer blend for 2 mins
Incubate 21 days 4°C
Plate 0.1 ml on SS and MacConkey agar
Inoculate 0.1 ml in 10 mls Selenite F broth
Incubate 25°C, 48 hrs
EITHER
Select lactose negative colonies
SCREENING TESTS
TSI 25°C Overnight
LIA 25°C Overnight
Urea 25°C 24 hr
Motility 25°C 24 hr
36°C 24 hr
PRESUMPTIVE IDENTIFICATION TESTS
Oxidase
Lysine decarboxylase 36°C
Arginine dihydrolase 36°C
Ornithine decarboxylase 25°C; 36°C
Phenylalanine deaminase 36°C
Indole 36°C
CONFIRMED TESTS 36°C
Lactose
Maltose
Sucrose
Melibiose
Raffinose
Rhamnose
OPTIONAL TESTS
biotype
serotype

and BS agars increased when *Y. enterocolitica* was inoculated onto raw pork samples at 5°C for 10 days (data not presented). This finding led to the following study which was designed to investigate the growth of *Y. enterocolitica* inoculated on raw pork at 0, 5 and 10°C for 12 days.

In this experiment uninoculated samples were also included and incubated at test temperatures. Total counts of uninoculated raw pork were approximately 1.8×10^2 and 7.0×10^3 c.f.u./gram of meat at 0 and 12 days, respectively. Isolates comprised of Gram positive cocci that possessed catalase activity, Gram positive bacilli that were catalase negative and Gram negative bacilli that possessed the enzymes, oxidase and catalase.

Results of this study show clearly that large populations of viable cells of *Y. enterocolitica* developed on raw pork at refrigerated temperatures (Fig. 29). These findings are in close agreement with the results reported by Hanna *et al.* (1977). They found large increases in *Y. enterocolitica* counts on raw pork incubated at 7°C for 10 days. These observations are of public health significance since the average storage and retail shelf life of fresh pork is approximately 10-14 days at 5°C. Attention is now being directed towards controlled gas atmosphere storage for meat products, including pork, to extend the shelf life for a period of up to 30 days. Such developments would present an even greater public health hazard. Although previous research on the use of gas mixtures is not at all extensive, the use of modified gas atmospheres may offer a possible solution to this problem.

Two categories of fresh meat preservation through controlled atmosphere are under study: 1. atmosphere modification through the

packaging technique, and 2. enrichment of the shipping container or storage chamber atmospheres by the addition of carbon dioxide; carbon dioxide-oxygen blends; carbon dioxide-carbon monoxide blends or carbon dioxide-oxygen-nitrogen blends.

Studies to investigate the effects of such gas mixtures on the growth of *Y. enterocolitica* must get underway immediately. Success in such a venture would probably be very helpful when proposed future standards or guidelines associated with the prolonged storage of fresh meats are outlined.

In the identification of *Y. enterocolitica* colonies isolated from pork, the API 20E microtube system was utilized. This system provided a rapid and convenient method for quick identification of *Y. enterocolitica* isolates. Restanio *et al.* (1979), in their report, supported the use of the API 20E system for the identification of *Y. enterocolitica* strains. They found that this system correctly identified 100% of both typical and atypical strains of *Y. enterocolitica* while the Minitek system identified only 80% of the same strains. Misidentified strains were atypical. The results of the present study therefore support the use of the API 20E system for rapid identification of *Y. enterocolitica*.

CONCLUSIONS

In evaluating this study, three major conclusions can be made:

1. *Y. enterocolitica* can grow well at refrigerated temperatures in laboratory media and on raw pork;
2. *Y. enterocolitica* can be isolated by direct plating on Violet Red Bile Glucose agar and incubating at 5°C for 10 days;
3. The ability of *Y. enterocolitica* to grow well at refrigerated temperatures, taken in conjunction with marketing and distribution systems for pork, present and proposed, would appear to merit further immediate investigation.

LITERATURE CITED

ABRAMOVITCH, H., BUTAS, C. Septicemia due to *Yersinia enterocolitica*. Canadian Medical Association Journal 109:1112, 1973.

AHLQVIST, J., AHVONEN, P., RÄSÄNEN, J.A., WALLGREN, G.R. Enteric infection with *Yersinia enterocolitica*. Large pyroninophilic cell reaction in mesenteric lymph nodes associated with early production of specific antibodies. Acta Pathologica et Microbiologica Scandinavica, Section A 79:109, 1971.

AHO, K., AHVONEN, P., LASSUS, A., SIEVERS, K., TIILIKAINEN, A. HL-A 27 in reactive arthritis: A study of *Yersinia* arthritis and Reiter's disease. Arthritis and Rheumatism 17:521, 1974.

AHVONEN, P. Human yersiniosis in Finland. I. Bacteriology and Serology. Annals of Clinical Research 4:30, 1972a.

AHVONEN, P. Human yersiniosis in Finland. II. Clinical features. Annals of Clinical Research 4:39, 1972b.

AHVONEN, P., ROSSI, T. Familial occurrence of *Yersinia enterocolitica* infection and acute arthritis. Acta Paediatrica Scandinavica Supplementum 206:121, 1970.

AHVONEN, P., SIEVERS, K., AHO, K. Arthritis associated with *Yersinia enterocolitica* infection. Acta Rheumatologica Scandinavica 15:232, 1969.

AHVONEN, P., THAL, E., VASENIUS, H. Occurrence of *Yersinia enterocolitica* in animals in Finland and Sweden. *In*: "*Yersinia Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973. 135 p.

ALBERT, G., LAFLEUR, L. *Yersinia enterocolitica* in children: Presentation of 24 cases observed at Hospital Sainte-Justine (Montreal). Canadian Journal of Public Health 62:70, 1971.

ALONSO, J.M., BERCOVIER, H., DESTOMBES, P., MOLLARET, H.H. Pouvoir pathogène expérimental de *Yersinia enterocolitica* chez la souris athymique (Nude). Annales de Microbiologie 126B:187, 1975.

ANON. Panel Proceedings - Microbiological Problems in Food Preservation by Irradiation. Vienna: International Atomic Energy Agency, 1966.

ARVASTSON, B., DAMGAARD, K., WINBLAD, S. Clinical symptoms of infection with *Yersinia enterocolitica*. Scandinavian Journal of Infectious Diseases 3:37, 1971.

ASAKAWA, Y., AKAHANE, S., KAGATA, N., *et al.* Two community outbreaks of human infection with *Yersinia enterocolitica.* Journal of Hygiene 71:715, 1973.

BERGSTRAND, C.G., WINBLAD, S. Clinical manifestations of infection with *Yersinia enterocolitica* in children. Acta Paediatrica Scandinavica 63:875, 1974.

BLACK, R.E., JACKSON, R.J., TSAI, T., *et al.* Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. New England Journal of Medicine 298:76, 1978.

BOTTONE, E.J. *Yersinia enterocolitica*: A panoramic view of a charismatic microorganism. Chemical Rubber Company: Reviews in Microbiology 5:211, 1977.

BOTTONE, E.J., CHESTER, B., MALOWANY, M., ALLERHAND, J. Unusual *Yersinia enterocolitica* isolates not associated with mesenteric lymphadenitis. Applied Microbiology 27:858, 1974.

BUCHANAN, R.E., GIBBONS, N.E. (Eds.). Bergey's Manual of Determinative Bacteriology, 8th Ed., Williams and Wilkins, Baltimore, 1974.

CARTER, P.B., COLLINS, F.M. Experimental *Yersinia enterocolitica* infection in mice: Kinetics of growth. Infection and Immunity 9:851, 1974.

CHESTER, B., STOTZKY, G. Temperature-dependent cultural and biochemical characteristics of rhamnose-positive *Yersinia enterocolitica.* Journal of Clinical Microbiology 3:119, 1976.

COLEMAN, M.B. *Bacterium enterocoliticum.* Annual Report Division of Laboratories and Research, New York State Department of Health: 68. Albany, N.Y., 1950.

DANIELS, J.J., GOUDZWAARD, C. Enkele stammen van een op *Pasteurella pseudotuberculosis* gelijkend niet geidentrificieerd species, geisoleerd bis knaadieren. Tijdschrift voor Diergeneeskunde 64:213, 1965.

DARLAND, G., EWING, W.H., DAVIS, B.R. The biochemical characteristics of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis.* Department of Health, Education and Welfare. Publication 75: 8294. Centre for Disease Control, Atlanta, Ga., 1974.

DELORME, J., LAVERDIÈRE, M., MARTINEAU, B., LAFLEUR, L. Yersiniosis in children. Canadian Medical Association Journal 110:281, 1974.

DIAZ, R. Antigenic relationship of *Yersinia enterocolitica* Type 9 with other Gram-negative species. *In*: "*Yersinia, Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973. 157 p.

DOMARADSKIJ, I., MARCHENKOV, V., SHIMANJUK, N. New data obtained in comparative studies of plague agent and related organisms. *In*: "*Yersinia, Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973. 2 p.

EMODI, A.S., LECHOWICH, R.V. Low temperature growth of type E *Clostridium botulinum*. Spores. 2. Effects of solutes and incubation temperature. Journal of Food Science 34:82, 1969.

ESSEVELD, H., GOUDZWAARD, C. On the epidemiology of *Y. enterocolitica* infections: Pigs as the source of infections in man. *In*: "*Yersinia, Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973. 99 p.

FEELEY, J.C., LEE, W.H., MORRIS, G.K. *Yersinia enterocolitica*. *In*: Compendium of Methods for the Microbiological Examination of Foods", Edited by M. Speck, American Public Health Association, Washington, D.C., 1976. 351 p.

FREDERIKSEN, W. A study of some *Yersinia pseudotuberculosis*-like bacteria ("*Bacterium enterocoliticum*" and "*Pasteurella* X"). Proceedings of the 14th Scandinavian Congress of Pathology and Microbiology, Oslo, 1964. 103 p.

GREENWOOD, J.R., FLANIGAN, S.M., PICKETT, M.J., MARTIN, W.J. Clinical isolation of *Yersinia enterocolitica*: Cold temperature enrichment. Journal of Clinical Microbiology 2:559, 1975.

GUTMAN, L.T., OTTESEN, E.A., QUAN, T.S., NOCE, P.S., KATZ, S.L. An inter-familial outbreak of *Yersinia enterocolitica* enteritis. New England Journal of Medicine 288:1372, 1973.

HAGEN, R.G., LASSEN, J., BERGE, L.N. Erysipelas-like disease caused by *Yersinia enterocolitica*. Scandinavian Journal of Infectious Diseases 6:101, 1974.

HANNA, M.O., STEWART, J.C., CARPENTER, Z.L., VANDERZANT, C. Development of *Yersinia enterocolitica*-like organisms in pure and mixed cultures on different Bismuth Sulfite agars. Journal of Food Protection 40:676, 1977a.

HANNA, M.O., STEWART, J.C., ZINK, D.L., CARPENTER, Z.L., VANDERZANT, C. Development of *Yersinia enterocolitica* on raw and cooked beef and pork at different temperatures. Journal of Food Science 42: 1180, 1977b.

HÄSSIG, A., KARRER, J., PUSTERLA, F. Über Pseudotuberculose biem Menschen. Schweizerische Medizinische Wochenschrift 79:971, 1949.

HIGHSMITH, A.K., FEELEY, J.C., MORRIS, G.K. *Yersinia enterocolitica*: A Review of the Bacterium and Recommended Laboratory Methodology. Public Health Laboratory Publication 14(4):253, 1977.

HOLT, J.G. (Ed.). The Shorter Bergey's Manual of Determinative Bacteriology, 8th ed, Williams and Wilkins, Baltimore, 1977.

HUBBERT, W.T. Yersiniosis in mammals and birds in the United States. Case reports and review. American Journal of Tropical Medicine and Hygiene 21:458, 1972.

INGRAM, M., MACKEY, B. Inactivation by cold. *In*: "Inhibition and Inactivation of Vegetative Microbes", Edited by F.A. Skinner and W.B. Hugo, Academic Press, London, 1976. 111 p.

JACKSON, H. Temperature relationships of *Vibrio parahaemolyticus*. *In*: "International Symposium on *Vibrio parahaemolyticus*", Edited by T. Fujino, G. Sakaguchi, R. Sakazaki and V. Takeda. Saikon Publishing Co. Ltd., Japan, 1973. 139 p.

JACOBS, J.C. *Yersinia enterocolitica* arthritis. Pediatrics 55:236, 1975.

KAPPERUD, G. *Yersinia enterocolitica* in small rodents from Norway, Sweden and Finland. Acta Pathologica et Microbiologica Scandinavica; Section B, Microbiology and Immunology 83:335, 1975.

KAPPERUD, G., JONSSON, B. *Yersinia enterocolitica* in brown trout (*Salmo trutta* L) from Norway. Acta Pathologica et Microbiologica Scandinavica, Section B, Microbiology and Immunology 84:66, 1976.

KEET, E.E. *Yersinia enterocolitica* septicemia. Source of infection and incubation period identified. New York State Journal of Medicine 74:2226, 1974.

KNAPP, W., THAL, E. A simplified antigenic schema for *Yersinia enterocolitica* (Syn. *Pasteurella* X) based on biochemical characteristics. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene erste Abteilung Originale. Reihe A 223:88, 1973a.

KNAPP, W., THAL, E. Differentiation of *Yersinia enterocolitica* by biochemical reactions. *In*: "*Yersinia*, *Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973b. 10 p.

KNORRING, J. VON., PETTERSSON, T. Haemolytic anaemia complicating *Yersinia enterocolitica* infection. Report of a case. Scandinavian Journal of Haematology 9:149, 1972.

LAFLEUR, L., MARTINEAU, B., CHICOINE, L. *Yersinia enterocolitica*. Aspects biologiques, épidémiologiques et cliniques de 67 cas

observes à L'Hôspital Sainte-Justine (Montreal-Canada). Union Medicale du Canada 101:2407, 1972.

LAITINEN, O., TUUHEA, J., AHVONEN, P. Polyarthritis associated with *Yersinia enterocolitica* infection. Clinical features and laboratory findings in nine cases with severe joint symptoms. Annals of the Rheumatic Diseases 31:34, 1972.

LASSEN, J. *Yersinia enterocolitica* in drinking-water. Scandinavian Journal of Infectious Diseases 4:125, 1972.

LAWRENCE, M.R., TING, S.K., NEILLY, S. Furuncle caused by *Yersinia enterocolitica*. Canadian Medical Association Journal 112:1289, 1975.

LEE, W.H. Two modified media for the isolation of *Yersinia enterocolitica*. Abstracts of the Annual Meeting of the American Society for Microbiology, 1975. 202 p.

LEE, W.H. An assessment of *Yersinia enterocolitica* and its presence in foods. Journal of Food Protection 40(7):486, 1977a.

LEE, W.H. Two plating media modified with Tween 80 for isolating *Yersinia enterocolitica*. Applied and Environmental Microbiology 33:215, 1977b.

MASSHOFF, W. Eine neuratige form der mesenterialen lymphadenitis. Deutsche Medizinische Wochenschrift 78:532, 1953.

MATCHES, J., LISTON, J. Low temperature growth of *Salmonella*. Journal of Food Science 33:641, 1968.

MATCHES, J.R., LISTON, J. Effects of incubation temperature on the salt tolerance of *Salmonella*. Journal of Milk and Food Technology 35:39, 1972a.

MATCHES, J.R., LISTON, J. Effect of pH on low temperature growth of *Salmonella*. Journal of Milk and Food Technology 35:49, 1972b.

MICHENER, H.D., ELLIOTT, R.P. Minimum growth temperatures for food-poisoning, fecal-indicator and psychrophilic microorganisms. Advances in Food Research 13:349, 1964.

MOLLARET, H.H. Un domaine pathologique nouveau: L'infection a *Yersinia enterocolitica*. Annales de Biologie Clinique 30:1, 1972.

MOLLARET, H.H., DESTOMBES, P. Les germe "X" en pathologie humaine. Presse Medicale 72:2913, 1964.

MOLLARET, H.H., GUILLON, J.C. Contribution a l'etude d'un nouveau groupe de germes (*Yersinia enterocolitica*) proches du bacille de Malassez et Vignal. II. Pouvoir pathogène expérimental. Annales

de l'Institut Pasteur 109:608, 1965.

MOLLARET, H.H., OMLAND, T., HENRIKSEN, S.D., *et al.* Les septicémies humaines à *Yersinia enterocolitica.* A propos de dix-sept cas recents. Presse Medicale 79:345, 1971.

MORRIS, G.K., FEELEY, J.C. *Yersinia enterocolitica*: a review of its role in food hygiene. Bulletin of the World Health Organization 54:79, 1976.

NICOLLE, P., MOLLARET, H., BRAULT, J. Recherches sur la lysogénie la lysosensibilité, la lysotypie et la serologie de *Yersinia enterocolitica. In*: "*Yersinia, Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973. 54 p.

NILÉHN, B. Studies on *Yersinia enterocolitica* with special reference to bacterial diagnosis and occurrence in human actue enteric disease. Acta Pathologica et Microbiologica Scandinavica Supplementum 206:1, 1969.

OHYE, D.F., CHRISTIAN, J.H., SCOTT, W.J. Influence of temperature on the water relations of growth of *Clostridium botulinum* Type E. *In*: "Botulism", Proceedings of the Fifth International Symposium of Food Microbiology, Moscow, Edited by M. Ingram and T. Roberts, Chapman and Hall, London, 1966. 136 p.

OLSON, J.C. Jr. U.S. Regulatory administration for control of micro-biological health hazards in foods. *In*: "Toxic Micro-organisms", Edited by M. Herzberg, Proceedings of the First U.S.-Japan Conference, United Stated Department of the Interior, Washington. 1970. 398 p.

OLSOVSKY, Z., OLSAKOVA, S., CHOBOT, S., SVIRIDOV, V. Mass occurrence of *Yersinia enterocolitica* in two establishments of collective care of children. Journal of Hygiene, Epidemology, Microbiology and Immunology 19:22, 1975.

PATERSON, J.S., COOK, R. A method for the recovery of *Pasteurella pseudotuberculosis* from faeces. Journal of Pathology and Bacteriology 85:241, 1963.

QUAN, T.J., MEEK, J.K., TSUCHIYA, K.R., HUDSON, B.W., BARNES, A.M. Experimental pathogenicity of recent North American isolates of *Yersinia enterocolitica.* Journal of Infectious Diseases 129:341, 1974.

RABSON, A.R., HALLETT, A.F., KOORNHOF, H.J. Generalized *Yersinia enterocolitica* infection. Journal of Infectious Diseases 131:447, 1975.

RABSON, A.R., KOORNHOF, H.J. *Yersinia enterocolitica* infections in South Africa. South African Medical Journal 46:798, 1972.

RABSON, A.R., KOORNHOF, H.J., NOTMAN, J., MAXWELL, W.G. Hepatosplenic abscesses due to *Yersinia enterocolitica*. British Medical Journal 4:341, 1972.

RESTAINO, L., GRAUMAN, G.S., McCALL, W.A., HILL, W.M. Evaluation of the Minitek and API 20E Systems for identification of *Yersinia enterocolitica*. Journal of Food Protection 42(2):120, 1979.

RITTER, D.B., GERLOFF, R.K. Deoxyribonucleic acid hybridization among some species of the Genus *Pasteurella*. Journal of Bacteriology 92:1838, 1966.

RUSTIGAN, R., STUART, C. Decomposition of urea by *Proteus*. Proceedings of the Soceity for Experimental Biology and Medicine 47:108, 1941.

SAEBO, A. Some surgical manifestations of mesenterial lymphadenitis associated with infection of the *Yersinia enterocolitica*. Acta Chirurgica Scandinavica 140:655, 1974.

SCHIEVEN, B.C., RANDALL, C. Enteritis due to *Yersinia enterocolitica*. Journal of Pediatrics 84:402, 1974.

SCHMIDT, C.F., LECHOWICH, R.V., FOLINAZZO, J.F. Growth and toxin production by type E *Clostridium botulinum* below 40°F. Journal of Food Science 26:626, 1961.

SCHLEIFSTEIN, J.I., COLEMAN, M.B. An unidentified microorganism resembling *B. lignieri* and *Past. pseudotuberculosis*, and pathogenic for man. New York State Journal of Medicine 39:1749, 1939.

SCHLEIFSTEIN, J.I., COLEMAN, M.B. *Bacterium enterocoliticum*. Annual Report Division of Laboratories and Research, New York Department of Health, Albany, 1943. 56 p.

SEGNER, W.P., SCHMIDT, C.F., BOLTZ, J.K. Effect of sodium chloride and pH on the outgrowth of spores of type E *Clostridium botulinum* at optimal and suboptimal temperatures. Applied Microbiology 14:49, 1966.

SMITH, J.E., THAL, E. A taxonomic study of the Genus *Pasteurella* using a numerical technique. Acta Pathologica et Microbiologica Scandinavica 64:213, 1965.

SNEATH, P.H., COWAN, S.T. An electro-taxonomic survey of bacteria. Journal of General Microbiology 19:551, 1958.

SOLEM, J.H., LASSEN, J. Reiter's disease following *Yersinia enterocolitica* infection. Scandinavian Journal of Infectious Diseases 3:83, 1971.

SONNENWIRTH, A.C. Bacteremia with and without meningitis due to *Yersinia enterocolitica*, *Edwardsiella tarda*, *Comamonas terrigena* and *Pseudomonas maltophila*. Annals of the New York Academy of Sciences, Article 2 174:488, 1968.

TALBOT, J.M., SNEATH, P.H. A taxonomic study of *Pasteurella septica*, especially of strains isolated from human sources. Journal of General Microbiology 22:303, 1960.

THAL, E. Untersuchungen über *Pasteurella pseudotuberculosis*. Thesis. Berlingska, Boktrycheriet, Lund, Sweden, 1954.

THATCHER, F.S., CLARK, D.S. Specifications for media. *In*: "Micro-organisms in Foods: 1. Their significance and methods of enumeration", University of Toronto Press, Toronto, 1975. 171 p.

TOIVANEN, P., TOIVANEN, A., OLKKONEN, L., AANTAA, S. Hospital outbreak of *Yersinia enterocolitica* infection. The Lancet 1:801, 1973.

TOMA, S. Survey on the incidence of *Yersinia enterocolitica* in the Province of Ontario. Canadian Journal of Public Health 64:477, 1973.

TOMA, S., DEIDRICK, V.R. Isolation of *Yersinia enterocolitica* from swine. Journal of Clinical Microbiology 2:478, 1975.

TOMA, S., LAFLEUR, L. Survey on the incidence of *Yersinia enterocolitica* infection in Canada. Applied Microbiology 28:469, 1974.

TOMA, S., LOIR, H., QUINN-HILL, M., SHER, N., WALKER, W.A. *Yersinia enterocolitica* infection: Report of two cases. Canadian Journal of Public Health 63:433, 1972.

TSUBOKURA, M., OTSUKI, K., ITAGAKI, K. Studies on *Yersinia enterocolitica*. I. Isolation of *Y. enterocolitica* from swine. Japanese Journal of Veterinary Science 35:419, 1973.

VANDEPITTE, J., WAUTERS, G., ISEBAERT, A. Epidemiology of *Yersinia enterocolitica* infections in Belgium. *In*: "*Yersinia, Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973. 111 p.

VAN LOGHEM, J.J. The classification of the plague-bacillus. Antonie van Leeuwenhoek: Journal of Microbiology and Serology 10:15, 1944.

WAUTERS, G. Contribution a l'etude de *Yersinia enterocolitica*. Ph.D. Thesis. Vander, Louvain, Belgium, 1970.

WAUTERS, G., Le MINOR, L., CHALON, A.M., LASSEN, J. Supplément au schéma antigénique du "*Yersinia enterocolitica*". Annales de l'Institut Pasteur 122:951, 1972.

WAUTERS, G., MOLLARET, H.H. Un nouveau cas d'infection humaine a *Yersinia enterocolitica*. Revue belge de Pathologie et de Medecine experimentale 31:328, 1965.

WETZLER, T.F. Animal diseases transmissible to man, pseudotuberculosis. *In*: "Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections", 5th ed., Edited by H.L. Bodily and E.L. Updyke. American Public Health Association, Washington, D.C., 1970. 449 p.

WINBLAD, S. Studies on Serological Typing of *Yersinia enterocolitica*. Acta Pathologica et Microbiologica Scandinavica Supplementum 187: 115, 1967.

WINBLAD, S. Erythema nodosum associated with infection with *Yersinia enterocolitica*. Scandinavian Journal of Infectious Diseases 1:11, 1969.

WINBLAD, S. The clinical panorama of human *Yersinia enterocolitica*. *In*: "*Yersinia*, *Pasteurella* and *Francisella*", Proceedings of the International Symposium, 1972 (Vol. 2, Contributions to Microbiology and Immunology), Edited by S. Winblad, S. Karger, Basel, 1973. 129 p.

WINBLAD, S. Arthritis associated with *Yersinia enterocolitica* infections. Scandinavian Journal of Infectious Diseases 7:191, 1975.

WINBLAD, S., NILÉHN, B., JONSSON, M. Two further cases, bacteriologically verified, of human infection with "*Pasteurella* X" (Syn. *Yersinia enterocolitica*). Acta Pathologica et Microbiologica Scandinavica 67:537, 1966. .

ZEN-YOJI, H., MARUYAMA, T. The first successful isolations and identification of *Yersinia enterocolitica* from human cases in Japan. Japanese Journal of Microbiology 16:493, 1972.

ZEN-YOJI, H., MARUYAMA, T., SAKAI, S., KIMURA, S., MIZUNO, T., MOMOSE, T. An outbreak of enteritis due to *Yersinia enterocolitica* occurring at a junior high school. Japanese Journal of Microbiology 17:220, 1973.

ZEN-YOJI, H., SAKAI, S., MARUYAMA, T., YANAGAWA, Y. Isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from swine, cattle, and rats at an abattoir. Japanese Journal of Microbiology 18:103, 1974.

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